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14. ABSTRACT

Clonal proliferation of plasma cells within the bone marrow (BM) affects local cells, such as mesenchymal stromal cells (MSCs), leading to osteolysis and fatality in multiple myeloma (MM). Consequently, there is an urgent need to find better mechanisms of inhibiting MM growth and osteolytic lesion development. To meet this need and accelerate clinical translation, better models of MM within the BM are required. We developed a clinically-relevant, 3D myeloma BM co-culture model that mimics bone cell-cancer cell interactions within the bone microenvironment. The co-culture model and clinical samples were utilized to investigate myeloma growth, osteogenesis inhibition, and myeloma-induced abnormalities in MM-MSCs. This platform demonstrated myeloma support of capillary-like assembly of endothelial cells and cell adhesion mediated-drug resistance (CAM-DR). Also, distinct normal donor (ND)- and MM-MSC miRNA signatures were identified and used to uncover osteogenic miRs of interest for osteoblast differentiation. More broadly, our 3D platform provides a simple, clinically-relevant tool to model cancer growth within the bone, useful for investigating skeletal cancer biology, screening compounds, and exploring osteogenesis. Our identification and efficacy validation of novel, bone anabolic miRs in MM opens the floodgate for novel approaches to cancer. We also further developed the OcnCre/iDTR model and discovered a novel bone marrow adipose tissue (BMAT) phenotype. We then explored reasons for this phenotype, and found evidence that sclerostin derived from osteocytes could potentially be one of the osteokines driving BMAT accumulation. We explored the use of metformin to decrease BMAT. Lastly, we found that using an anti-sclerostin antibody, we were able to decrease tumor burden and increase bone, as well as decrease BMAT, suggesting that this antibody could have a 2-pronged attack on the BM in myeloma.

15. SUBJECT TERMS
Multiple Myeloma, Blood Cancer, Hematological Malignancy, Bone Metastasis, 3D Model, In vitro, silk scaffolds, osteogenic microRNAs, adipocytes.

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1. Introduction

MM and other osteolytic cancers are devastating cancers that, for most patients, have no cure to this day. They develop by hijacking the bone in what is known as a vicious cycle, creating a forward feedback loop with local Mesenchymal Stem Cells (MSCs) in the bone and inhibiting their ability to differentiate into bone cells (osteoblasts). This, combined with increased osteoclastic activity induced by MM cells, leads to devastating skeletal consequences (ie. pain, hypercalcemia, and fracture) and accelerated tumor growth[1,2]. MM patient derived MSCs (MM-MSCs) exhibit decreased proliferation and osteogenesis, an inability to repair osteolytic damage, and display great patient-to-patient heterogeneity in their ability to undergo differentiation and induce changes in MM cells[2–4]. The tumor BM microenvironment also supports tumor growth[5], induces chemoresistance and selects for tumor-initiating clones[6]. Over the last few years, the scientific research community has made great strides with the treatment of Multiple Myeloma (MM). In November of 2015, alone, three new MM drugs were approved, bringing the total number of FDA-approved drugs for MM from 7 up to 10, all of which were given "priority review" to speed their transition to patients, and 2 of which were considered "breakthrough" drugs. However, challenges still remain in treating myeloma, and especially in treating MM bone disease. Many of these new drugs are very costly, and while they work for certain patients, other patients do not respond at all. My research has aimed primarily at either targeting or using the bone microenvironment, or understanding the biology of the bone marrow (BM) to develop better cures for MM that will not depend on the mutations present. My research utilizes the unique properties, cell types, and biochemistry of the bone microenvironment, which is relatively consistent across patients, and hence may be a better target, to develop novel ways to stop MM growth and heal or reduce bone disease. Moreover, as patients are living longer with MM, due to these novel therapies, it becomes more important to treat the osteolysis that has been induced by MM, to reduce bone pain and the occurrence of painful fractures. *Therefore, over the past 2 years, I have been researching bone-cancer interactions, and have made a number of discoveries and developments that will contribute to a better understanding of how MM progresses and creates non-healing osteolytic punched-out lesions*. The work herein discusses the development of a realistic model of the abnormal BM seen in MM patients, which will greatly benefit translational research scientists. The research provides novel targets for anti-cancer treatment by helping to heal osteolytic lesions in cancer patients, and provides a new platform to study cancer-bone interactions. Also supported by this funding, bone-targeting novel nanoparticles were developed for targeting myeloma, using a natural biopolymer. The bone-targeting polymer-based nanoparticles were developed and validated in a pre-treatment *in vivo* setting in an MM1S mouse model[7]. Lastly, we investigated an an anti-sclerostin antibody, which builds bone and appears to reduce bone marrow adiposity, and found that it is a successful treatment in mice for myeloma-induced bone disease in a collaboration with researchers at the Garvan Institute, Sydney, Australia.

2. Keywords

Multiple Myeloma, Blood Cancer, Hematological Malignancy, Bone Metastasis, 3D *In vitro* model, silk scaffolds, osteogenic microRNAs, nanoparticles, osteolysis, sclerostin.

3. Overall Project Summary

Progress and Accomplishments & Results

Statement of Work	Actual Tasks Performed
Task 1 (Months 1-8)	
a) Seek regulatory approval from IRB	All approvals were sought and obtained.
b) Make Silk Scaffolds (Carmen Preda)	Silk scaffolds formulated and obtained from Tufts.
c) Isolate MSCs from bone marrow	Marrow MSCs collected from IRB-approved sources.
d) Generate Models of osteogenesis in 3D scaffold cultures.	This has been designed, optimized and performed with microCT, confocal imaging, H&E and alizarin red staining for validation.
e) Assess GFP+/Luc+ MM1S, OPM2, other cells, on scaffolds in response to osteoprogenitors using	This has been done with MM1S, OPM2, RPMI cells, as well as primary patient cells, and imaged with confocal imaging. (Figure

confocal imaging	3,4,). We also saw that primary MM cells grow on these (Figure 11)
f) Remove stromal and MM1S cells from mono- and co-cultures, separate using FACS (fluorescence-activated cell sorting) or MACS™ magnetic sorting beads, assess both cell types for proteomics, mRNA expression and miRNA expression.	This has been done using FACS (Figure 5) and cells were assessed for miRNA, mRNA and proteomics. Focus remained on stromal cells, as the cultures of myeloma cells alone did not grow well and so were hard to compare to the co-culture systems. Also, due to low numbers of cells obtained after flow sorting, only miRNA analysis was deeply pursued further and done in replication.
g) Validate changes in stroma and in myeloma using immunohistochemisty, flow cytometry, and/or western blotting	This was done using NanoString molecular biology analysis and q-RT-PCR of miRNA. Also, Clinical Samples of MSCs from Normal donor (ND) and Myeloma patients, (as well as ND-MSCs co-cultured with MM1S MM cells) were analyzed for changes in miRNA and mRNA. As we began focusing on the changes we saw in miRNA expression in MM vs ND MSCs, (Table 1 and Figure 6, 9), IHC, WB and Flow cytometry were not pursued.
g.i) Validate results in osteoprogenitors regarding bone differentiation	This was done using the confocal microscopy and histology for alizarin red staining, to show that indeed MM cells do inhibit osteogenesis and patient MM-MSCs don't differentiate correctly[8].
g.ii) Characterize functional differences between normal and myeloma osteoprogenitors samples using assays for proliferation	This has been performed using cell counting of both clinical samples (ND- and MM-MSCs), and in the 3D Model with vs without MM1S myeloma cells using direct cell counting (Figure 1, 2).
g.iii) Culture ND-MSCs with and without MM cells for different time regimens to determine if changes induced in MSCs are reversible and how long it takes for these changes to arise	We cultured MM-MSCs and ND-MSCs and determined that the inhibited ability to differentiate, and proliferate is retained in MM-MSCs even after isolation from bone marrow and passaging. These cells become quickly senescent and hence it appears that the effects of myeloma cells on the stromal cells are not reversible.
g.iv) Explore methods of reversing the effects of cancer on osteoprogenitors such as knockdown or overexpression techniques, pharmaceuticals, neutralizing antibodies, or cell therapies.	This was done using miRvana mimics and inhibitors *in vitro* to increase and decrease the expression of certain microRNAs that differed in ND- vs MM-MSCs from clinical samples and from the 3D co-culture system (Table 1, Figure 6). We found that by adding miR199a to MM-MSCs we were able to induce osteogenic differentiation, but inhibiting the overexpressed miRs did not have this same effect. Thus miR199a appears to be a novel osteogenic miR that is a new target for therapy (Figure 7,8) In a related project, I used our findings to study if increased osteogenesis of osteoprogenitors inhibits tumor growth, using a pre-treatment with bortezomib-loaded, alendronate-conjugated PEG-PLGA nanoparticles which we published[7]. The effects of bortezomib on hMSCs are shown in Figure 10.
Task 2: Develop an *in vivo* model from osteo-transgene mice and Vk*myc MM cells. (Months 1-15) (Aim 1b and 2b).	
a) Begin ACURO and IACUC approval for a total of 90 mice	I wrote an entirely new IACUC protocol for this purpose, which was approved, and obtained ACURO approval for this work.
b) Isolate GFP+CD138+ MM cells from Vk*myc mice using FACS	The cells we obtained were not GFP+, but we did obtain CD138+ Vk*Myc cells as a cell line from Dr. Martha Chesi, rather than the Vk*Myc Transgenic mice themselves. We are currently still attempting to label these with Luciferase and GFP, which is challenging since they don't grow *in vitro*. Cells are being expanded *in vivo* subcutaneously, and we are also trying still to get an in vitro cell line to grow, but this made transfection of these cells nearly impossible. Still, we have shown (Figure 18) the ability to track Vk*Myc growth using ELISA, IgG M-spikes, survival, and IHC in the bone marrow (where we did see some tumor cells accumulate)
c) Inject MM cells into osteo-transgene mice and assess disease course.	We have initiated these studies and have preliminary data with OcnCre/iDTR mice (Figure 12) However, we found that these mice have significant changes in their immune system, which was

	also recently published by Dr. Scadden[9], (Figure 13). Thus, we decided to further instigate this phenotype before rushing into injecting tumor cells, due to the fact that many cell types were changing at once in this model. Still, we were able to get the depletion and bone loss with Diphtheria Toxin injection in these mice (Figure 15), but we also saw that this was very lot-dependent, mouse responses varied greatly, and we had no fast readout to tell how the bones were responding to DT until the endpoint, so progress has been moving slowly with this model. The Vk*Myc cells most often went to the spleen (see Figure 16), making it challenging to see how modulation of the bone marrow altered MM homing and growth in the marrow, as many mice died before having significant bone metastasis. We have tried to make a bone-passage cell line (Figure 17) and are still working on this. As we were investigating this, we discovered that the Osteocalcin-Cre KO mice had a profound, later proven significant, increase in BMAT- Bone Marrow Adipose Tissue (Figure 14). This had never been reported on before. This therefore further complicated the use of this model to study the effects of osteo-progenitors on MM growth in the BM, since the increases in adipose tissue may also affect this process. As we found little knowledge in the literature about bone marrow adipose, especially in terms of how it may relate to cancer in the marrow, we went on to investigate this, so that we could better pull out the effects of BMAT from Osteocytes/Osteoblasts in these models. To do this, we performed a few experiments without MM and made some exciting discoveries, such as the fact that treating mice with Metformin (Figure 23) was able to decrease bone marrow adipose tissue. We aim to build on this now to study MM in the BM and effects of BMAT as well as Osteocytes and Osteoblasts in my new lab.
i) Refine model variables such as numbers of Vk*myc-MM cells injected, injection site, and timeframes.	We have done this (Figure 18) and are still in the process of perfecting the model and isolating a bone-metastatic clone, because Vk*Myc Cells often went to the spleen rather than the bone marrow. Still, we have shown that bone metastasis and osteolysis occurs with VkMyc tail-vein injection. Tumor cell expansion works well when injected subcutaneously in Matrigel. Timeframes as dependent on number of cells injected is still being studied, as the Vk*Myc cells are very heterogeneous. We are also still exploring differences in metastatic sites based on different methods of implantation (ex: sub-Q, within a donor mouse femur). We are trying to derive a Luc+ *in vitro* Vk*Myc cell line.
ii) If necessary, develop a NOD/SCID model where osteoprogenitor cells from osteo-transgene mice are isolated based on fluorescence (and hence osteogenic status) and co-injected with Luc+ MM1S cells. (Aim 1b Alternative)	We have not had to pursue this alternative direction since it appears that Vk*Myc cells are growing well in the Black6 background.
2) Task 3: Utilize the *in vivo* model of Task 2 to address biological questions: 1) how do different types of osteoprogenitors affect MM cells? and 2) how do MM cells affect different types of osteoprogenitors? 3) What new targets and therapies does this work suggest? (Months 15-21) (Aim 1b and 2b). a) Identify effects of osteoprogenitor cells on MM1S. (Aim 1b) i) Assess disease progression and bone marrow homing using bioluminescent imaging, *in vivo* fluorescent confocal microscopy of the calvaria, bone marrow flow cytometry, survival, mouse	As planned, we began to look at the effects of different osteoprogenitors on MM growth. However, in the Take-One-Out Strategy using osteocalcin-KO mice is more complicated than expected, discussed above, The removal of osteocalcin itself may also be causing issues in these mice. As stated above, the Vk*Myc cell line appeared to be very heterogeneous, with some mice dying much sooner than others, so we are trying to select for a more homogenous clone that is bone homing using in vitro expansion attempts and in vivo bone-passaging. This is still in progress. We have been successful in genotyping and breeding the mice, injecting them with Vk*Myc and assessing tumor burden, but have not been able to stay specifically that the

weight, and IHC. ii) Determine differential disease progression and preferential bone homing based on osteoprogenitor status. Once osteoprogenitor subtypes are identified as preferentially tumor-supportive *in vitro* and *in vivo*, lentiviral or RNAi knockdown or overexpression methods will be used to elucidate underlying mechanisms of osteoprogenitor support of MM disease progression.	removal of osteoblasts/osteocytes increases or decreases MM progression. To understand this better, we performed in vitro analyses with MSCs differentiated to different extents (3 days, 1 week, 2 weeks, 3 weeks), and found great heterogeneity in the effects of differentiated MSCs compared to their control undifferentiated controls. In general, MM1S appeared to be less supported by the more-differentiated stromal cells (Figures 24). For 2ai) Using the MM1S model, we were able to validate that osteo-progenitors have anti-myeloma effects both in vitro and in vivo using an anti-sclerostin antibody in a collaboration with Dr. Croucher and Dr. McDonald. (Figures 19-20). This has demonstrated that modulating the bone microenvironment with bone anabolic agents not only has the potential to slow tumor growth, but also significantly increases bone volume per total volume (BV/TV), bone strength, and trabecular and cortical thickness, suggesting great clinical utility to combat cancer-induced bone disease for MM and other bone metastasis patients. For 2aii) We are trying to understand the reasons why, in the MM1S model we were able to see decreased tumor burden in mice treated with the bone-anabolic (anti-sclerostin) antibodies. This could be due to a direct effect of osteoblasts on MM cells such as the induction of apoptosis, cell-cycle arrest/senescence, or quiescence, as suggested by a recent publication by our colleagues.[10] We are now performing more in vitro studies with osteocytes (OCY454 cells) and osteoblasts (MC3T3 and hFOB cells) to determine the true effects of bone cells on MM cells. We are also exploring if the bone-building anti-sclerostin antibodies may also have had an inhibitory effect on MM cells through a different mechanism: their modulation of BMAT. This is based on our observation that there was less marrow adipose in the anti-sclerostin treated mice compared to controls, for both tumor-bearing mice and naïve mice. Thus, we are investigating if SOST (sclerostin) may be an adipogenic agent, suggesting a second method of action for this drug. We are also working with clinicians in Maine and Massachusetts, and pharmaceutical companies to try to take this work into the clinic to translate it to MM patients.
b) Identify effects of MM1S on osteoprogenitor cells. (Aim 2b) i) Utilize live *in vivo* calvaria confocal imaging, dual-energy X-ray absorptiometry for bone density, and serum analysis for ALP or osteocalcin, (osteogenesis markers) and serum TRAP 5b (bone resorption markers).	I developed the *in vivo* calvaria confocal imaging protocols for our lab and performed some preliminary work with this technique[11,12], but as we became more interested in aim 2a, we did not spend very much time trying to understand how MM1S cells affect osteoprogenitors. We did, however, demonstrate that they slow growth rates and differentiation in vitro, and induce changes in mRNAs and microRNAs in hMSCs, that could be targeted to normalize MSCs, as we published[8].
c) Perform endpoint analysis including histology for bone health, IHC for characterization of osteoprogenitor subtypes within endosteal and periosteal surfaces, and microCT/X-ray analysis for osteolytic formation.	We have done preliminary studies, (Figure 25) to see osteolysis in the femur of MM1S-bearing mice. It is not easy to use these modalities to examine osteoprogenitors in these models, but we now have a GFP-Ocn mouse that we plan to use in confocal microscopy to specifically examine interactions between tumor cells and Ocn+ cells. We have also demonstrated osteolysis in the MM1S model can be reversed in the femur, tibia and vertebrae (cortical and trabecular bone) with treatment with Anti-sclerostin antibodies, using microCT (Figure 20) and static histomorphometry (data still coming). We aim to repeat this study and better characterize this before we submit the work for publication, hopefully in April 2016. We have also done preliminary studies in this area with our Sost-KO mice and we are trying to better characterize the osteolysis, and protection

	from that in low-sclerostin conditions, with these models.
d) Test therapies for their ability to correct and normalize osteoprogenitors, enable bone healing, and reduce tumor burden in mouse models and assess results using techniques described above (BLI, histology, in vivo confocal, bone marrow flow cytometry, microCT, serum biomarkers, mouse weight, etc.) i) Explore decoy ligands, exogenous proteins, ECM proteins, small molecule inhibitors or alternative delivery mechanisms in Aim 2 to normalize aberrant osteoprogenitors and impede stromal support of MM.	As described above, we have performed in vitro work using microRNA199a[8] and *in vivo* work using Sclerostin-neutralizing antibodies in this area (Figure 19, 20), based on epidemiological data showing increased sclerostin in MM patients vs. Healthy patients, and based on the biological action of sclerostin. This was performed with our collaborators, Dr. Michelle McDonald and Dr. Peter Croucher, in the Garvan Institute, Sydney Australia. We presented this at ASH[13] and ANZBMS in 2015. This treatment has proven to increase bone parameters and inhibit tumor burden in our MM1S model. We are now validating that work and reproducing it before submitting it for publication.
Task 4: Compile data, write manuscript, submit to high-impact journal and perform extra experiments potentially requested by reviewers. (Months 21-24).	I have done this; I published a first author manuscript in Blood[8] and co-first in PNAS[7] and also published 2 review articles based on this research. Figure 26, from one of these reviews, provides an over view of the work done during this project. More publications are under review and in preparation about this work.

Impact

Our work opened the door revealing the ability for microRNAs to affect osteogenesis, which could be a novel mechanism to heal osteolytic lesions and block tumors from growing in bone. Our work also demonstrated the ability to target the bone marrow to modify the bone before cancer has arrived, to make the microenvironment less hospitable to cancer cells, by using bortezomib-loaded bisphosphonate-conjugated nanoparticles. We developed novel technologies (3D Bone models, and bone-targeting nanoparticles), and novel results (specifically that microRNA-199a is able to induce osteogenic differentiation), and novel findings (that the bone can be modulated to make it less hospitable to cancer cells, and that microRNA levels are significantly different in MSCs from normal donors and myeloma patients). We are building on this work to better understand how cancer grows in the bone and how we can better stop its growth and destruction of the bone marrow. We are currently discussing performing clinical trials with bone-targeting nanoparticles with bortezomib to reduce off-target side effects associated with bortezomib. We also found that anti-sclerostin antibodies hold very good promise to inhibit MM or stop cancer-induced bone disease *in vivo*.

Other disciplines may also benefit from our work. Many other diseases beside MM may benefit from the miR199a and anti-sclerostin antibody, including other types of bone cancer, such as metastatic breast or prostate cancer, or other bone diseases where osteogenesis is inhibited, such as osteoporosis. We have demonstrated that stromal cells from cancer patients are abnormal and that one of the abnormalities is expression of a certain microRNA, and that by correcting (increasing) that microRNA, the stromal cell can regain its ability to undergo osteogenesis. Also, the novel, bone-targeting PEG-PLGA nanoparticles could be used clinically to target any cancer, or other disease, of the bone. Third, we report a new mechanism of action for the drug metformin, to inhibit bone marrow adipose tissue, which may be important for diabetic patients, especially obese diabetic patients, to understand how metformin may be working. Fourth, the discovery that bone marrow adipose increased in our OcnCre-iDTR mice suggests a molecular mediator regulating adipogenesis coming from osteocytes, and we believe that molecule be sclerostin, based on some preliminary work shown here. This could have impacts on any other metabolic or hematopoietic-disruption disease, as well as any bone-homing tumors.

Regarding an impact on technology transfer, it is possible that government research or industries will now start to research how they can target or deliver certain microRNAs to the bone marrow to affect bone strength and normalize the bone cells, which have been altered by cancer cells. Also, a start-up company called BIND may build on the bortezomib nanoparticle technology and may start to produce these nanoparticles in much higher quantities so that they can be used in more cancer trials and eventually, hopefully, in patients. Lastly, we are now working with a company called BioPact with their MGMR (medical grade molecular rebar) multi-walled carbon nanotubes, building off the PEG-PLGA nanotubes we developed in this project, and we hope to be able to see these be developed by the company for use in humans. We have currently received a 1 year sponsored

research agreement from BioPact to perform this research (Sept 2015-Sept 2016) and I have hired one post-doc with these funds to get this research going.

More globally, our research will potentially have impact on changing the way society sees diseases of the bone. If we are successful in showing that the bone microenvironment is able to foster or inhibit bone cancer growth, we may be able to empower individuals more into prevention of bone cancer, which is something that people currently do not think they are personally able to predict or change. If we can show that stronger bones are better able to protect against bone cancer, or that decreased adipose (systemic or local in the marrow) can decrease the progression of myeloma, this may change people's actions. We may change people's understanding of the need for personal prevention, exercise, diet and bone strength. We may thusly instill a sense that a person's own choices can have a very significant effect on their outcomes, prevent future cancers or inhibit osteolytic lesion development, but much of this work still needs to be carried out in the future to fully understand how and why stronger bones, or less bone adipose, could prevent myeloma growth. In sum, this could make the nation healthier, not just in terms of cancer, but also with ramifications in obesity, diabetes, and osteoporosis.

Changes/Problems: Nothing to Report

Participants & Other Collaborating Organizations

Name:	Michaela Reagan
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ORCID 0000-0003-2884-6481
Nearest person month worked:	24
Contribution to Project:	Dr. Reagan has performed and oversaw all work in this project.
Funding Support:	This award alone during the 2 year funding period.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - No/Nothing to Report.
- **What other organizations were involved as partners?**
  - **Organization Name:** Tufts University
  - **Location of Organization:** Medford, MA, USA
  - **Partner's contribution to the projec**t
    - **Financial support**: None
    - **In-kind support:** Silk Scaffolds made at Tufts Biomedical Engineering Dept staff by Mrs. Carmen Preda, in the lab of Dr. Kaplan, our collaborator.

  - **Organization Name:** Maine Medical Center Research Institute
  - **Location of Organization:** Scarborough, ME, USA
  - **Partner's contribution to the projec**t
    - **Financial support**: None
    - **In-kind:** Advice, consultation, collaboration on OcnCre/iDTR mouse work.

Data and Results

Although key data have been shown in the attached manuscripts, there was also substantial data not shown in the manuscripts or important to highlight here.

As part of Task 1, we validated first that we could culture MSCs with Myeloma cells. *In vitro* we found that MSCs significantly increased proliferation of myeloma cells using cell counting at days 0 and day 6 (Figure 1).

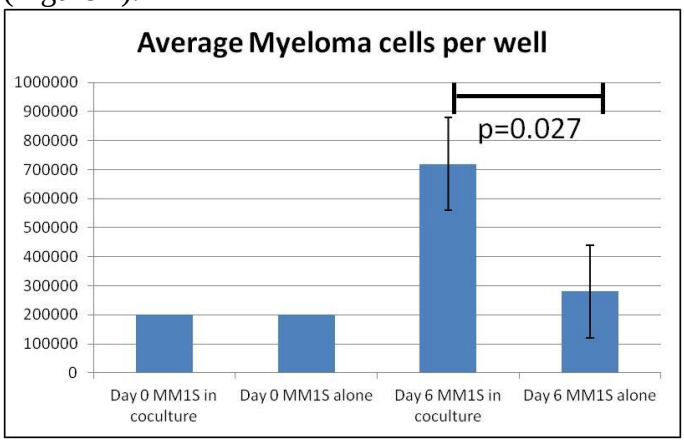

Figure 1: MSC induced proliferation of myeloma MM1S tumor cells.

We also verified this finding by looking at the tumor cells under a fluorescent microscope with representative images, as shown in Figure 2 below, from 3 different wells.

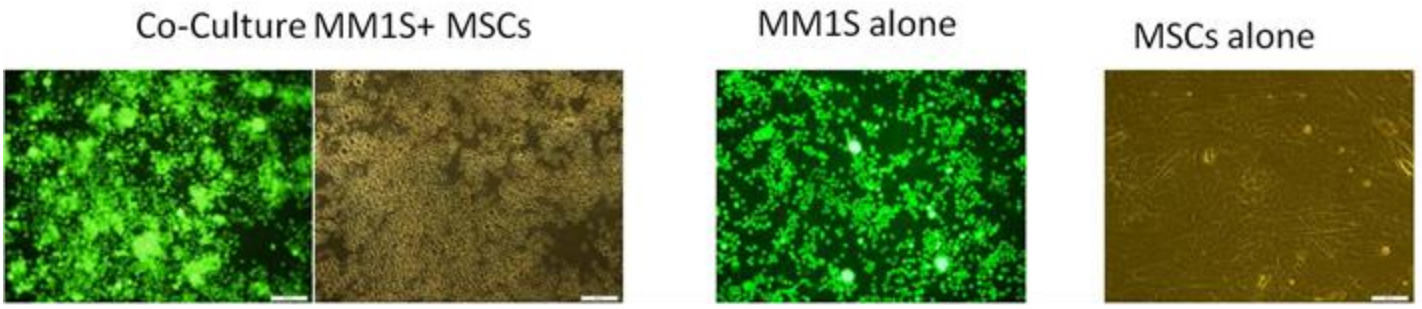

Figure 2: Increased MM1S proliferation and adhesion of MM1S to stroma and increased clumping over stroma. Concurrently, observed inhibited stromal growth, which was quantified by cell counting using nuclear staining (Hoescht), which was put into the Blood manuscript.

One of the major challenges of the long-term culture of myeloma cells with MSCs on the 3D model was cell labeling. The first dye we used to stain the MSCs for the experiment was the cell tracker dye "DiD". Unfortunately, this dye's fluorescent signal became diminished over time, with each cell replication, to the point that we were not able to produce very good images of the MSCs on the scaffolds by week 4, as shown in Figure 3 below. Our next step was to use a TurboRed (RFP)-containing plasmid packaged into a lentivirus to infect the cells and increase the signal. This allowed us to flow sort the cells from the scaffolds, and this is the data we used for further work and presented in the Blood manuscript.

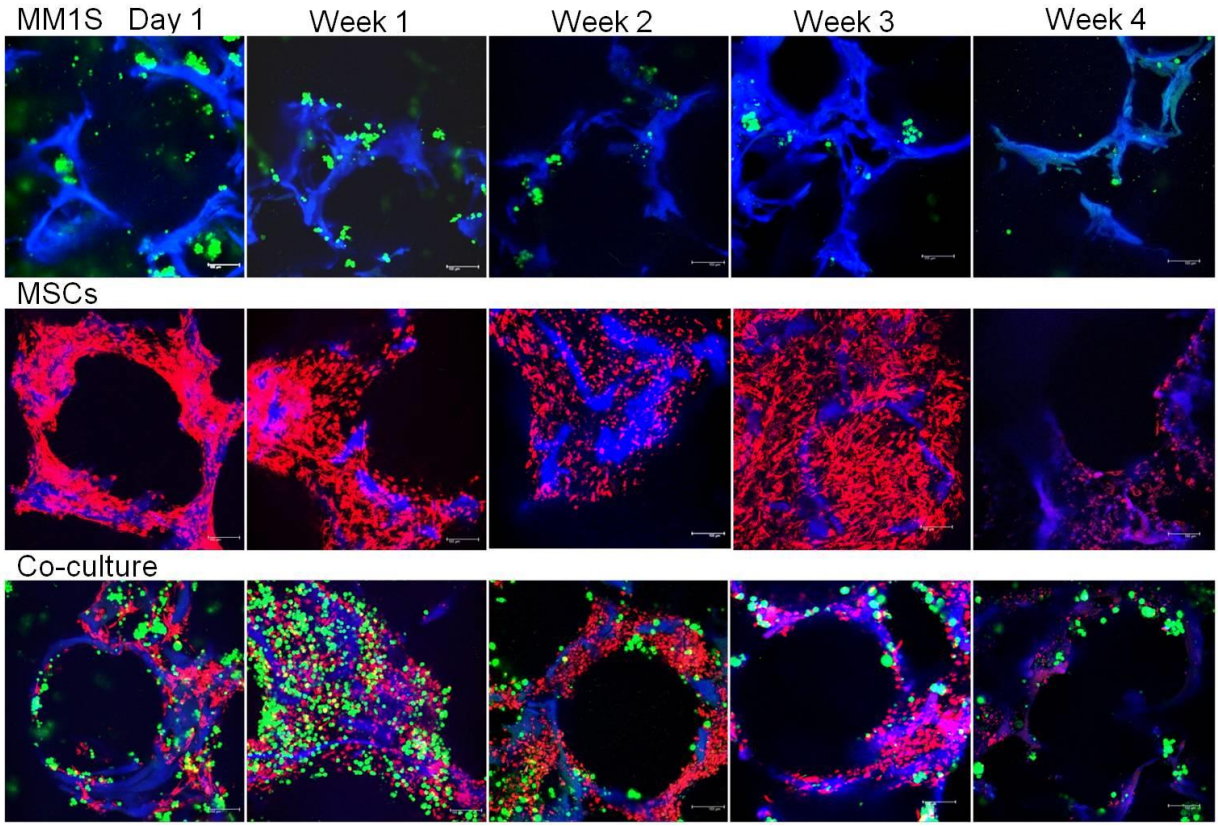

Figure 3: Silk Scaffolds imaged on confocal microscope from Day 1, and weeks 1, 2, 3 &4. MSCs are labeled red with DiD and myeloma cells are green and expressing GFP and luciferase. We determined the cell tracker dye (lipophilic DiD) would not be an acceptable labeling mechanism for MSCs over the long-term due to reduced signal over time.

We also initially attempted to simply use mechanical disruption of the 3D cultures to remove the myeloma cells from the scaffolds for Task 1, but we found that this would not help us to separate the cells because this process also washed away the MSCs that were on the scaffolds. This is shown in Figure 4. We then used FACS (Fluorescently-activated cell sorting) to separate the myeloma cells and the MSCs based on the different spectral properties of the cells. An example of this is shown in Figure 5.

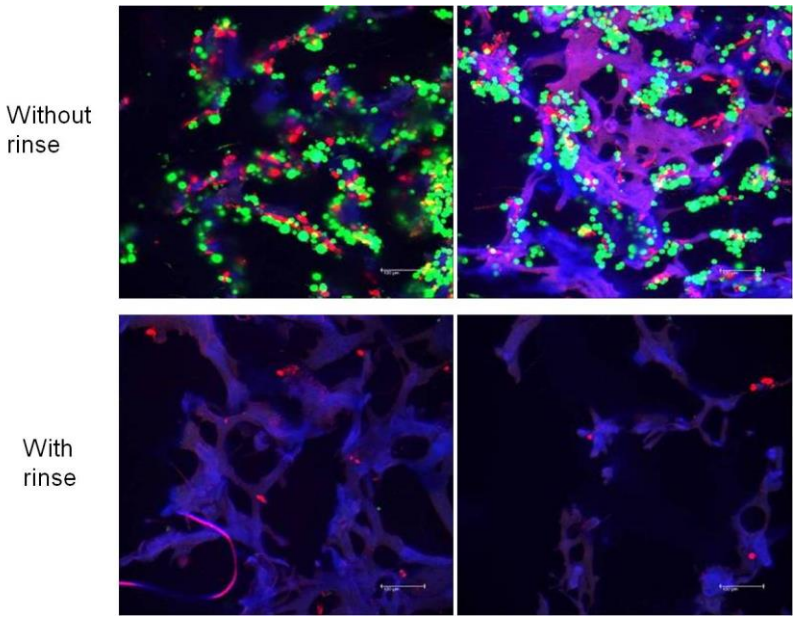

Figure 4: Silk Scaffolds (Purple), shown with MSCs (red) and with MM1S cells (green). Although the mechanical rinsing of scaffolds was able to remove almost all of the MM1S cells, the MSCs were also removed from the scaffold, making this an unreliable method for removing MM1S cells from scaffolds. From this, we decided to use rinsing, trypsinization, and FACS to separate the two cell types for further miRNA and mRNA analysis.

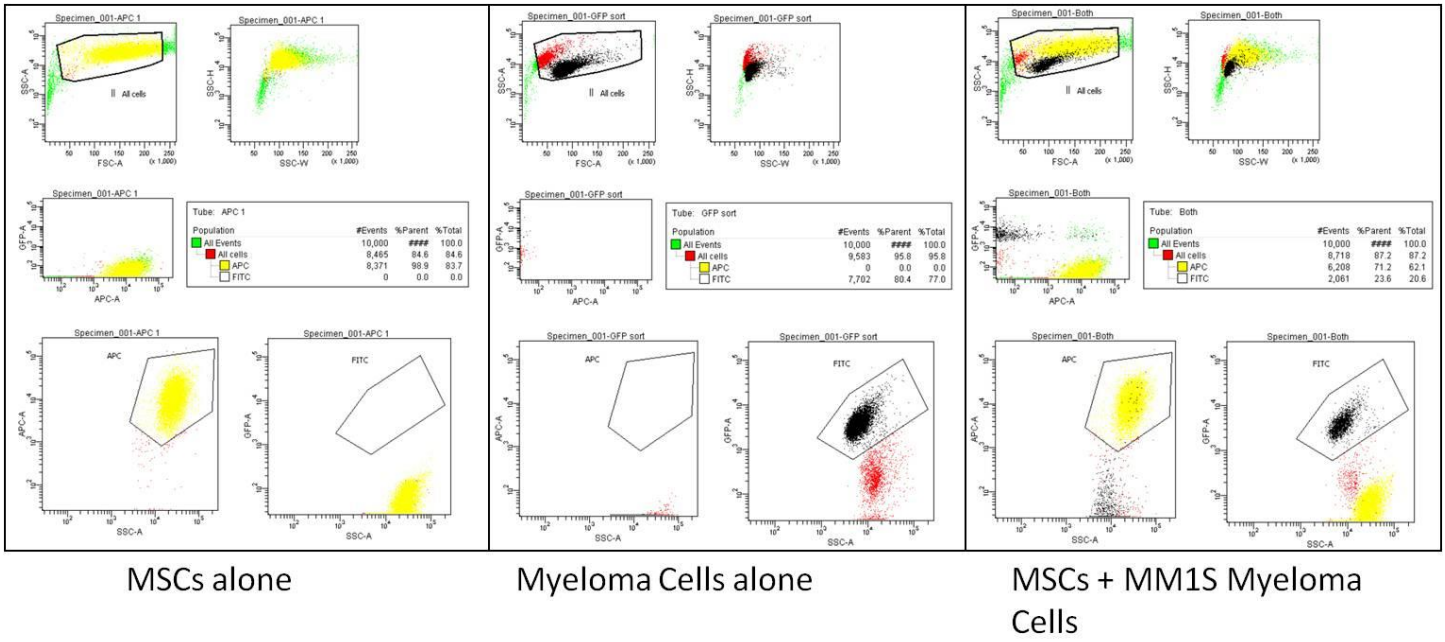

Figure 5: FACS (Fluorescently-activated cell sorting) of cells from scaffolds. MSCs alone, Myeloma cells alone or Myeloma plus MSCs are shown. This method was used to isolate and separate cells from scaffolds.

MicroRNAs were analyzed from patient samples (healthy vs myeloma stroma) and from the 3D Model stromal cells sorted from scaffolds (monocultured vs co-cultured with myeloma cells in osteogenic medium for 2 weeks) and all miRNA NanoString data was uploaded to the GEO database under accession number GSE60423. MicroRNAs identified as significantly different between ND-MSCs and MM-MSCs in patients (Figure 6A) and in the 3D model (Figure 6B) were summarized in Table 1 below and further investigated using miRNA mimics or miRNA inhibitors.

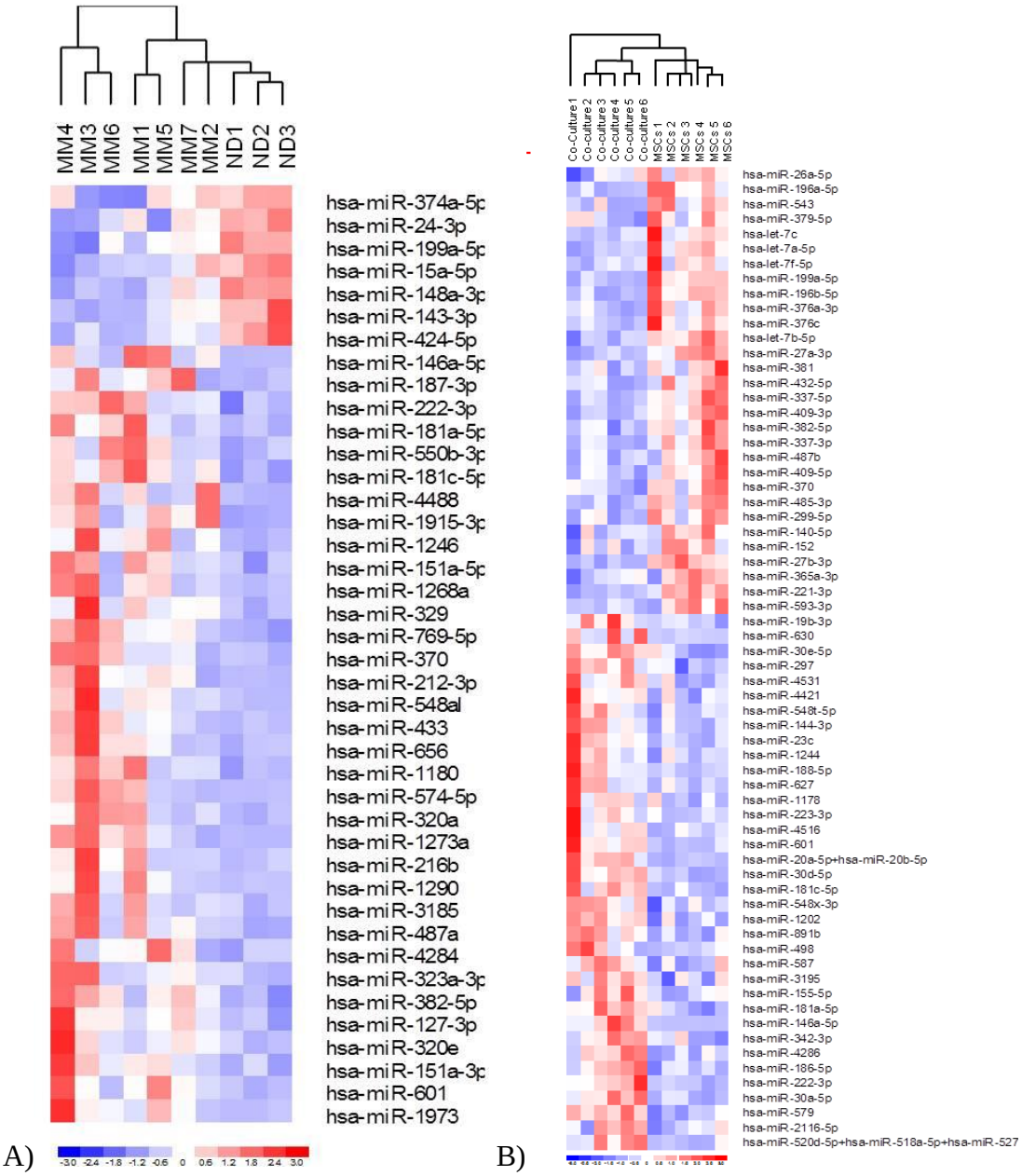

Figure 6: microRNA NanoString data is from patient samples (A, left) and the 3D model co-culture (B, right). MSCs were collected, lysed in Trizol, and processed for microRNAs using a Qiagen microRNeasy mini kit. MicroRNAs were then quantified using a nanodrop and expression of 800 microRNAs was analyzed using a Nanostring microRNA panel.

Table 1: **MicroRNAs altered in MSCs by Myeloma**. Six microRNAs were found to be similarly upregulated (5 miRs) or downregulated (1 miRs) in the 3D system (MSCs co-cultured with GFP^{+}MM1S vs. MSCs alone, after 2 weeks in co-culture in osteogenic, no-dexamethasone media) and in patient vs. normal samples (MM patient MSCs vs. normal donor MSCs)**.** Fold changes, fc≥1.5, $p<0.05$, n≥3, 2-tailed T-test, average expression>25 NanoString counts.

miRNA Name	Fold Change, 3D Model (MSCs in co-culture with MM1S vs Alone)	p-value, 3D Model (MSCs in co-culture with MM1S vs Alone)	Fold Change, MM vs ND MSCs	p-value, MM vs ND MSCs
hsa-miR-199a-5p	**-2.019**	**0.00086974**	**-1.917**	**0.00115665**
hsa-miR-181a-5p	**1.771**	**0.00362835**	**3.190**	**0.02106839**
hsa-miR-181c-5p	**2.135**	**0.00276756**	**3.078**	**0.00192591**
hsa-miR-222-3p	**2.152**	**0.00332040**	**1.821**	**0.01680615**
hsa-miR-601	**2.546**	**0.00738047**	**3.637**	**0.02449335**
hsa-miR-146a-5p	**17.175**	**0.00405939**	**15.353**	**0.02622689**

Those microRNAs that were increased in MM conditions (Table 1, bottom 5 microRNAs) were analyzed using inhibitors. However, the inhibitors did not affect functional osteogenesis of MSCs (Figure 7). In contrast, the mimics used to increase levels of hsa-miR-199a were able to significantly increase the osteogenic differentiation of MSCs (Figure 8)[8].

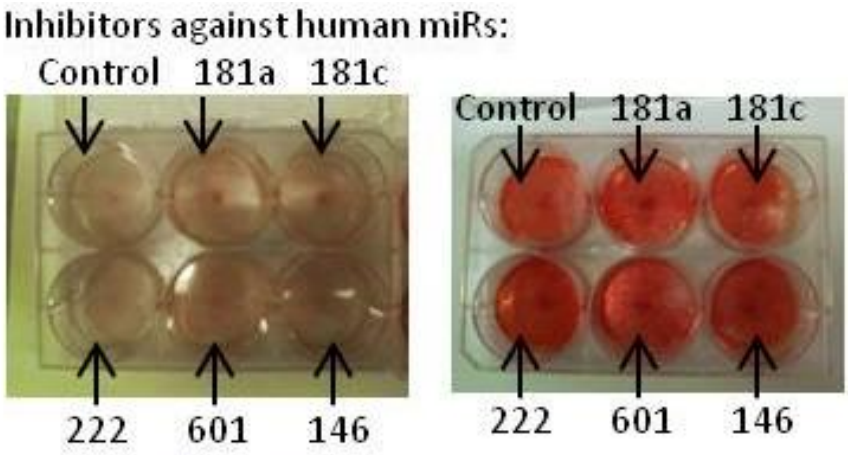

Figure 7: Inhibition of over-expressed microRNAs shows no effect. Representative Alizarin Red 6-well plates shown. The miRNAs identified as overexpressed in MM-MSCs (refer to Table 1) from 3D model and clinical samples was not able to show any functional change in osteogenic differentiation in MSCs and was not further pursued. Of note, the MSC donor on the right seemed to have high baseline osteogenic potential compared to most MM-MSC samples, perhaps due to treatments the patient was on. These microRNAs may be very important in explaining other differences, aside from osteogenesis, between normal MSCs and MM-MSCs and should be further investigated for the clinical implications of their altered expression in MM patients, as the effects may not be related to osteogenic alterations.

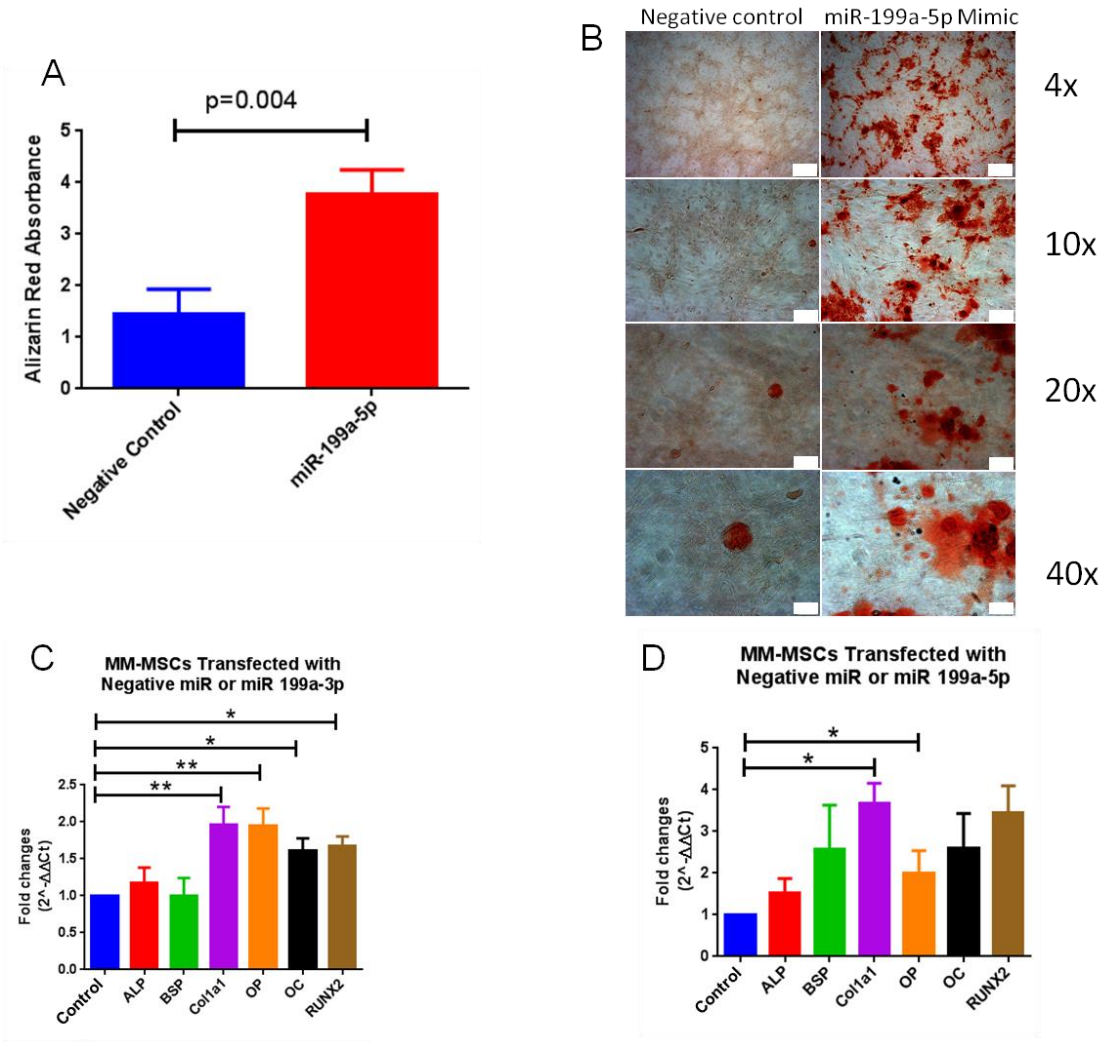

Figure 8: Osteogenic Induction microRNA 199a-5p. A) Quantification of Alizarin Red Staining of MSCs treated with negative control microRNA or miR-199a-5p mimics. B) Representative images of fixed samples of MSCs differentiated with Negative control microRNA mimics or miR-199a-5p mimics. C) qRT-PCR quantification of gene expression of osteoblast lineage mRNAs in MM-MSCs treated with miR-199a-3p or miR-199a-5p. (D) Alkaline phosphatase (ALP), integrin-binding sialoprotein (BSP), collagen type I alpha 1 (Col1a1), osteopontin (OP), osteocalcin (OC), and runt-related transcription factor 2 (RUNX2). E) 6-Well plates with miR199a mimics showed significant enhancement of osteogenic differentiation, which are seen as dark red wells, compared to control mimics, the alternating wells containing little stain.

As part of Task 1g, we also examined the differences between primary samples in terms of mRNA expression using a Nanostring panel of 230 cancer-associated genes in MM-MSCs and ND-MSCs (Figure 9). Of interest was the increased expression of certain cell cycle kinase inhibitors CDKN2A (cyclin-dependent kinase inhibitor 2A) and CDKN1A (cyclin-dependent kinase inhibitor 1A) in MM samples. The elevated expression of these genes may partially explain why we see inhibited proliferation of MM-MSCs and may be a novel target in the attempt to stimulate these cells to overcome senescence in myeloma patients and begin to differentiate into osteoblasts again.

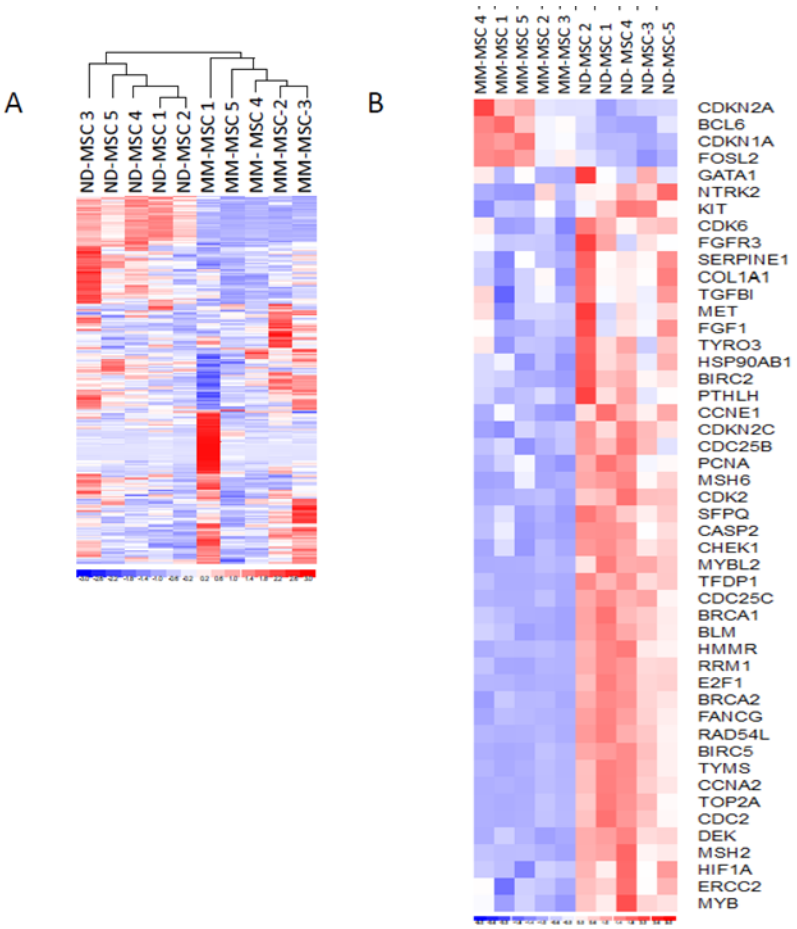

Figure 9: mRNA profiling of MM vs ND-MSCs. (a) Unsupervised clustering distinguishes MM- and ND-MSCs (passage 2) based on 230 cancer-related mRNAs (Nanostring analysis). (b) Of these, 49 mRNAs were significantly different ($p<0.05$, ≥ 1.3 fold change, MM- vs ND-MSCs).

Also as part of Task 1g.iv, we explored bortezomib, due to prior reports of anabolic action, and found that *in vitro* treatment of MSCs, and *in vivo* whole body treatment of mice, was able to induce osteogenic differentiation (Figure 10). This data was then used to build upon, and used to enhance a collaboration with MIT on bone-targeting nanoparticles for myeloma, which was published in PNAS 2014. This work with bortezomib-bone homing nanoparticles would not have been done without the methods and skills used and learned for the 3D model work that this grant supported.

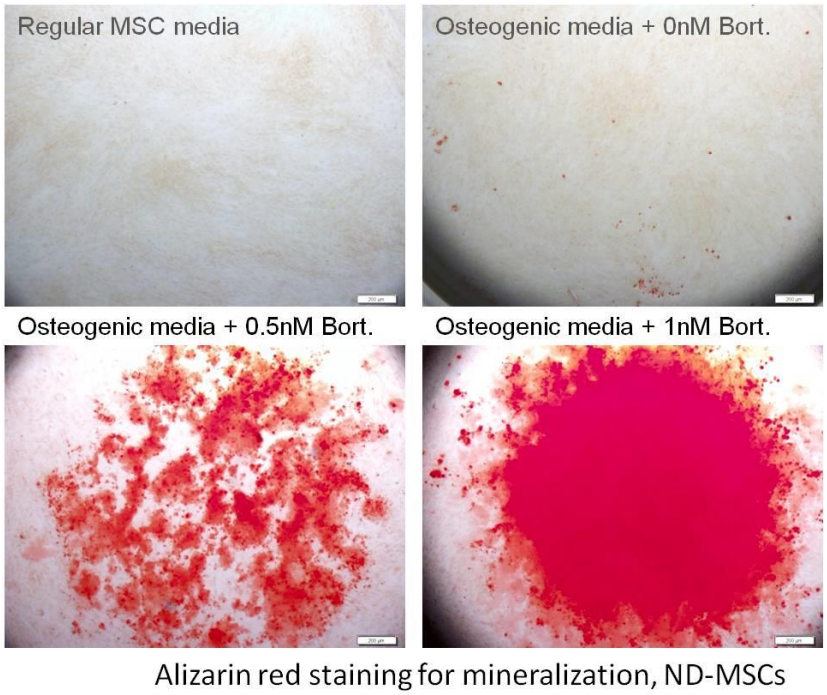

Figure 10: Alizarin Red staining after 1 week of differentiation demonstrates ability for bortezomib to increase osteogenesis of bone marrow-derived MSCs. Bortezomib treatment in early stages may prove be a novel tool to increase bone formation in MM patients and protect against osteolysis.

We have also cultured primary patient myeloma cells on the silk scaffolds (Figure 11) seeded with MSCs and we find that MM patient cells (CD138 bead selected) prefer to grow on MSCs rather than on naked silk scaffolds, similar to our findings of MM cell lines on scaffolds. Due to the nature of patient-to-patient tumor cell heterogeneity, we have decided, for our studies, to use myeloma cell lines for analysis of miRNA changes in MSCs, but future directions could use this 3D Model to make patient-specific bone marrow niche mimics for personalized drug or miRNA screens or drug resistance analysis.

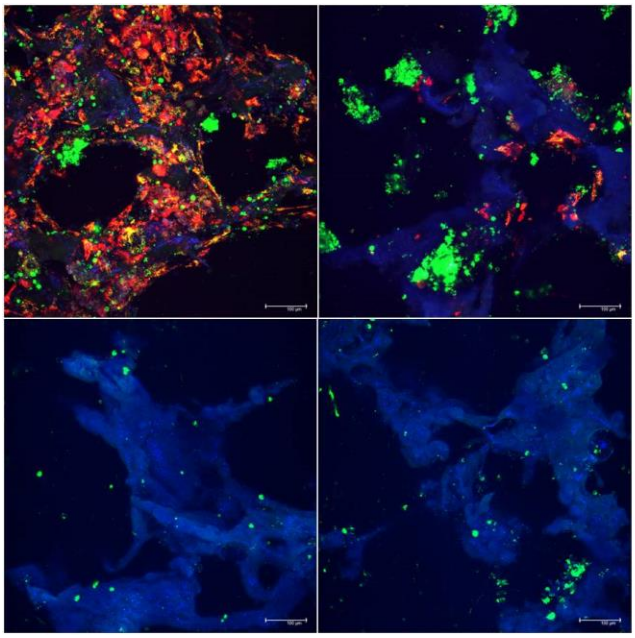

Figure 11: Confocal Imaging at Day 4 of Primary Patient MM cells (green) on scaffolds seeded with MSCs(red/orange) or on scaffolds alone (blue). MM cells show preference for growing on MSCs compared to the blank scaffolds.

As part of Task 2, we characterized the osteogenic transgene Ocn-Cre/iDTR mice (which have a knock-out of osteocyte and osteoblast cells) and have found that, during a 2 week treatment of the mice with diphtheria toxin (DT), mice are significantly smaller than their normal counterparts (Figure 12) and show alterations in their bone marrow hematopoetic cellular components. Mice breed well and are normal without DT injections. We saw an increase in the numbers of T-cells from .88% to 3.24% in the bone marrow immediately after the 2 week DT treatment period (Figure 13), as well as some other interesting immune changes that we are currently pursuing more deeply.

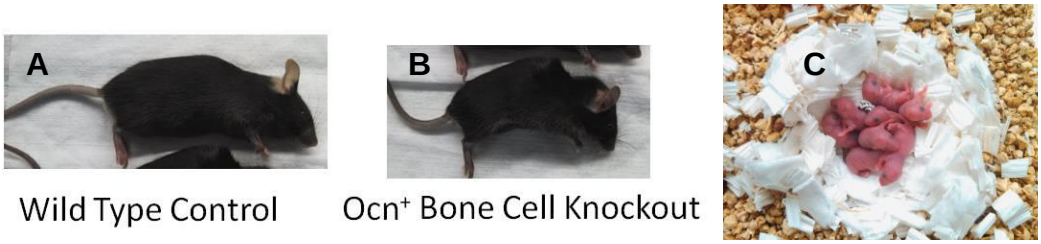

Figure 12: Representative Images of Wildtype (A) and Osteocalcin-Cre ;iDTR bone knockout mouse (B) show decreased weight, subcutaneous adipose, and a hunched posture in KO mice. C) Pups from the OcnCreiDTR transgenic mice when they are first born. There is no difference in OcnCre/iDTR mice and WT mice as pups or through adulthood, until Diphtheria Toxin is injected, at which point the mice begin to lose bone and weight as their osteocytes and osteoblasts begin to die.

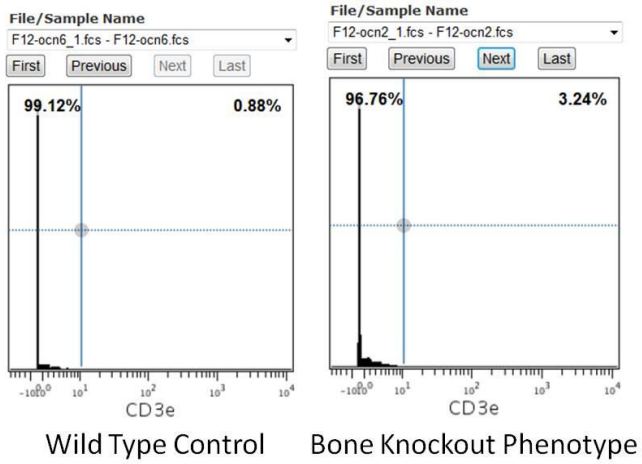

Figure 13: Representative CyTof bone marrow analysis of T-cell populations in Wild Type control mice (left) and Ocn+ Bone Cell Knockout mice (right). Using CyTof, many immune cells were found to be upregulated immediately after 2 weeks of DT treatments, but T-cells were found to be decreased 3 weeks after stopping DT (not shown), which was also reported and published by our collaborator, Dr. Scadden.[9] This further complicated the use of this model to study osteoprogenitor's effects on MM, as we know the immune system has effects on MM growth. Hence we are still working on how to best approach these extra complications of the model.

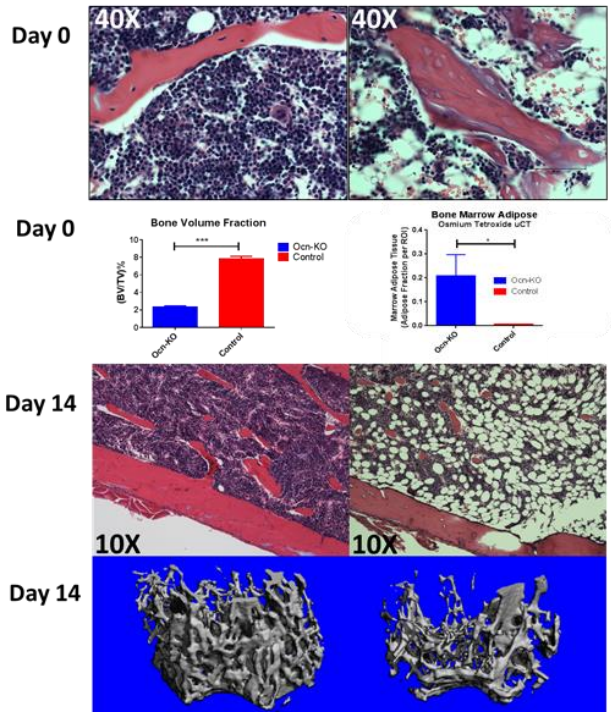

Figure 14: *In Vivo* Model of OcnCre/iDTR mice. Day 0 and Day 14 images from Control (left) and OcnCre/iDTR mice (right). Top: Day 0) Representative H&E of femur BM immediately after 2 weeks of DT treatment to remove osteocalcin+ cells demonstrated mostly empty osteocytic lacunae, decreased bone volume fraction and increased BMAT (quantified in graphs below). Bottom: Day 14) Representative H&E of femur BM 2 weeks after stopping DT treatment showsh decreased bone and increased BMAT remains. Representative µCT images demonstrate corresponding reduced trabecular bone.

We characterized the bone phenotype of the OcnCre/iDTR mice (Figure 14-15) and observed significant decreases in bone volume and bone cells as expected. Surprisingly, we saw increases in Bone Marrow Adipose Tissue (BMAT) in the mice. We were encouraged to follow this interesting finding, to understand why this occurs, so that we could better understand the model system to use with the Vk*Myc cells, and other diseases related to bone marrow adipose tissue.

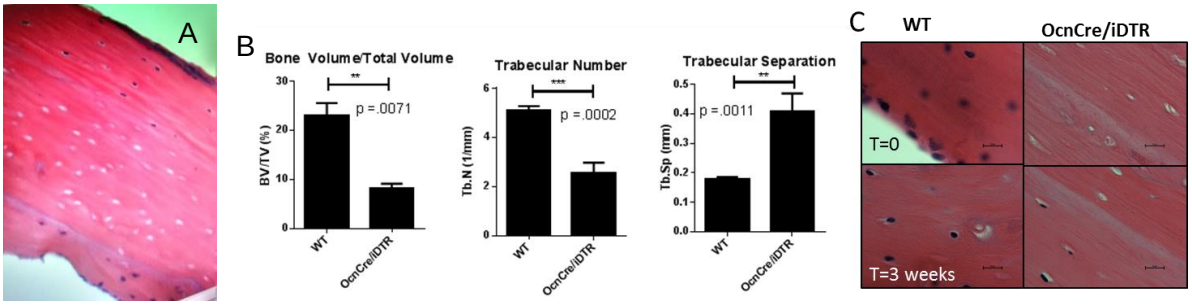

Figure 15: *In Vivo* transgenic model of Osteocalcin-Cre; inducible Diphtheria Toxin Receptor (OcnCre/iDTR) Black6 mice. A) H&E section shows osteocytes are gone, leaving vacant lacuna, 2 weeks after stopping a 2 week DT treatment. Cement lines are formed and new osteocytes in new bone (blue dots in pink cortical bone on the top and bottom edges). B) Quantification with uCT of these bones showed significant decreases in OcnCre/iDTR bone phenotype relative to WT controls in BV/TV (Bone Volume Fraction, or Bone Volume per Total Volume) and Trabecular Number, and increases in Trabecular Separation. C) H&E histology at 100x of mouse femur cortical region from WT (left) and OcnCre/iDTR (right) mouse bones. Elimination of osteocytes from bone matrix at T=0 (time point 0 days after DT injection), and a recovery of bone with new osteocytes appearing at T= 3 weeks (3 weeks after stopping DT injection) next to the dead region that remained free of osteocytes

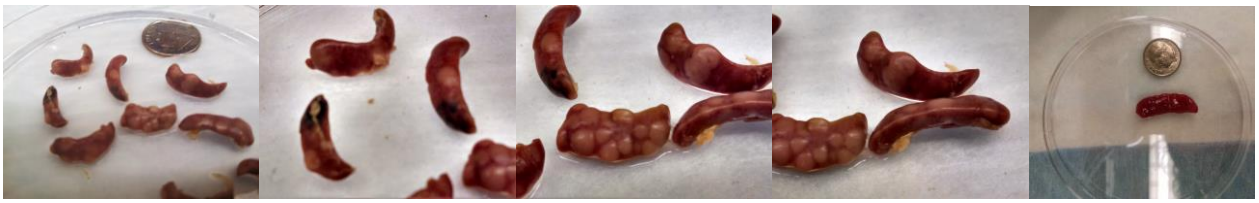

Figure 146: Vk*Myc Cells Metastasize to Spleen rather than Bone Marrow. These mice were injected with Vk*Myc MM cells and appeared to have spleen, rather than metastasis. We are still undertaking the mission of developing better bone-targeting MM Vk*Myc line in my new laboratory in Maine using intratibial direct injections of Vk*Myc cells.

In parallel, we characterized the Vk*Myc myeloma model and found that, after *i.v.* injection, these cells primarily go to the spleen and often caused death due to splenomegaly (Figure 16). We tried to isolate the cells that went to the bone marrow and passage these through C57/B6 mice (Figure 17) as well as immune-compromised mice, but as of yet we have not been able to isolate a strongly bone-metastatic clone of Vk*Myc MM cells. This is an ongoing research direction in the Reagan lab.

Figure 157: Mouse Used to Select Bone-Metastatic Vk*Myc Cells. This mouse was injected with Vk*Myc MM cells and appeared to potentially have bone metastasis to the skull that was causing hair loss, but he died from spleen metastasis and we were not able to isolate any bone metastatic cancer cells from him. We are still undertaking the mission of developing a better bone-targeting MM Vk*Myc line.

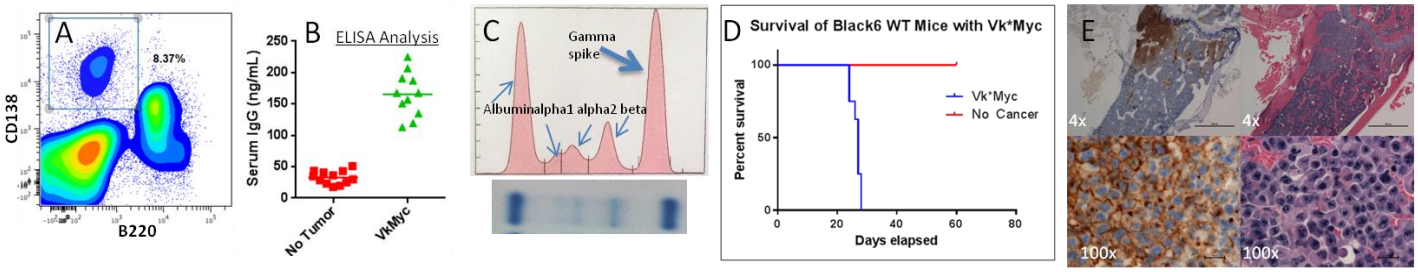

Figure 18: *In Vivo* model of Vk*Myc Myeloma Cell growth in B6 mice. A) Bone marrow flow cytometry on tumor-bearing mice showing that ~8.37% of bone marrow cells are tumor cells ($CD138^+$, $B220^-$ population) at 3 weeks after injection. B) Bone Marrow Serum ELISA for IgG also quantified tumor burden in Vk*Myc-injected mice. C) We observed monoclonal spike (M-spike) in serum protein electrophoresis in the gamma region, quantitatively demonstrating increased IgG antibodies at 3 weeks post Vk*Myc injection in mice. D) Survival of tumor-bearing mice. E) IHC and histology sections demonstrating tumor colonization in femoral bone marrow using CD138 (brown, left) and H&E (right) at 4x (scale=500µm) and 100x (scale=10µm) 3 weeks after Vk*Myc cell i.v. injection.

As an alternative avenue for exploring the effects of bone on MM, we also explored the use of anti-sclerostin antibodies from Novartis in an MM1S human xenograft myeloma model. We injected MM1S cells tail vein into Scid-Beige mice and then injected weekly treatments with anti-sclerostin antibodies until day 28, when we sacrificed the mice. We also performed bioluminescence imaging (BLI) on these mice to see if the bone-anabolic effects could also inhibit the tumor growth. Tumor burden was significantly decreased in these mice (Figure 18) and bone parameters were significantly increased in mouse tibia, femora and vertebrae (Figure 19), which we presented at ASH, 2015[13]. This is the first time an anti-myeloma effect has been observed in an *in vivo* model of MM.

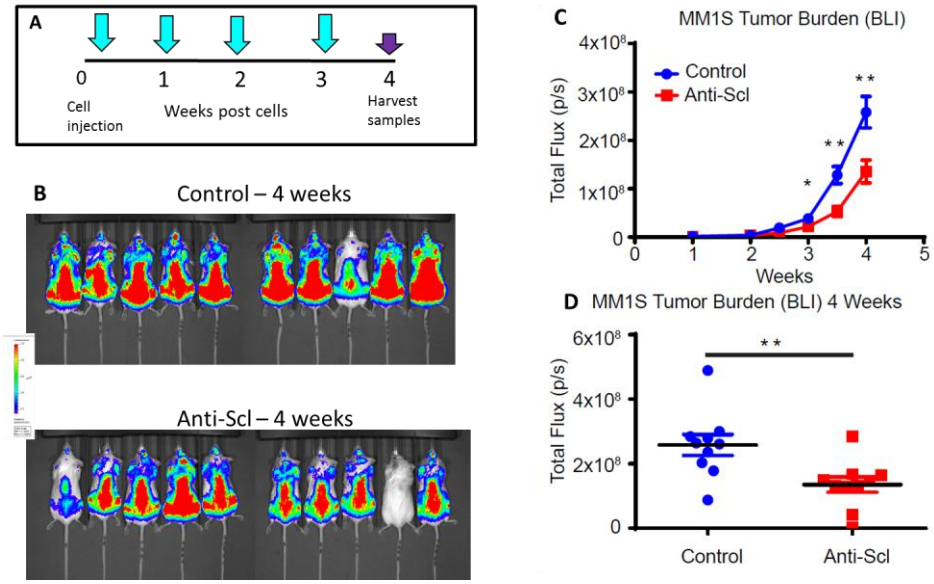

Figure 169: Anti-Sclerostin antibodies inhibit MM growth in SCID-beige mice. A) Experimental design. B) Representative mouse images of mice imaged longitudinally to follow the evolution of disease with BLI (bioluminescent imaging). C-D) Tumor burden was significantly decreased with Anti-Scl antibodies vs. Control. This data builds on the recent Nature Communications publication by Dr. Lawson, McDonald, Croucher and colleagues, demonstrating that osteoblasts can induce dormancy in MM tumor cells[10]. We are currently investigating the mechanisms behind the anti-tumor effects we observed, to see if this may be a dormancy or quiescence effect, and further exploring the roles of osteoblasts and bone in MM disease progression.

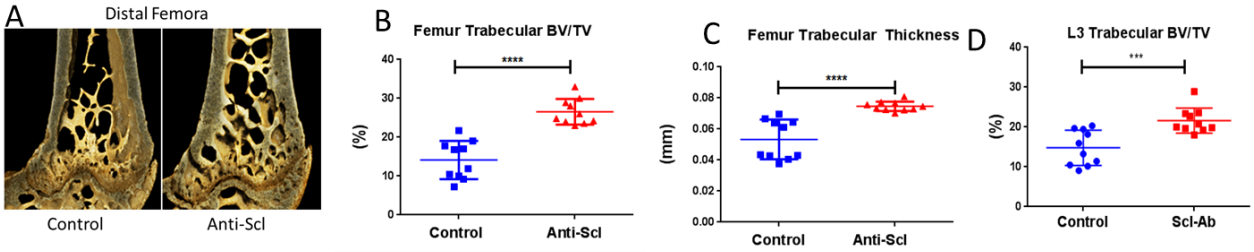

Figure 20: Representative reconstructions of µCT images of distal femora and quantification. A) Distal femur µCT data, also demonstrated that MM1S bearing mice had significantly increased femur trabecular BV/TV (bone volume per total volume), trabecular thickness, and L3 vertebrae BV/TV. and other bone parameters not shown, when treated with Scl-Abs. This result was also observed in tibia (not shown).

We also noticed in these mice that bone marrow adipose appeared to be decreased in tumor bearing mice treated with anti-sclerostin antibodies vs tumor bearing mice along, and in naïve mice plus anti-sclerostin antibodies vs naïve mice plus controls (Figure 21). We then hypothesized that sclerostin may be a pro-adipogenic osteokine and that by decreasing sclerostin we could decrease adiopogenesis. This has proven to be true in the 3T3-L1 mouse pre-adipocyte cell line as well as human and mouse MSCs, assessed by Oil-Red-O staining (Figure 22). We also found that metformin is another way to modulate the bone marrow adipose tissue, which is interesting because diabetics on metformin have a decreased risk of progression to MM compared to diabetic not on metformin (Figure 23). Of course, this is a retrospective study, but it still suggests that potentially metformin could reduce MM growth by reducing bone marrow adiposity[14]. The potential for metformin as an anti-myeloma treatment has also been suggested by some in vivo mouse MM model data[15].

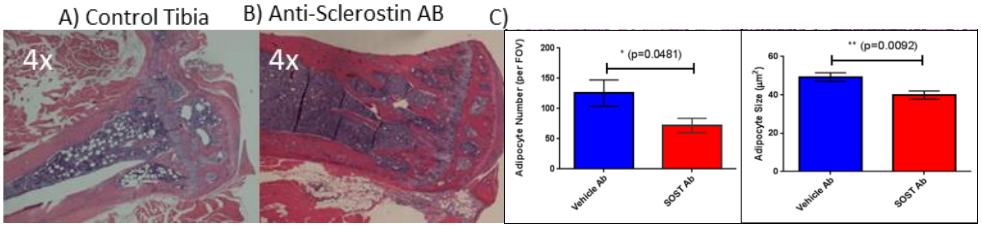

Figure 21: SOST-Abs decrease BMAT. Representative H&E images of tibial metaphysis from B6 mice treated with Buffer control (A) and Anti-Sclerostin Antibodies (B) weekly i.v. for 5 weeks. C) Tibia treated with Anti-sclerostin antibodies had significantly less adiposity than controls based on bone marrow adipocyte size and number.

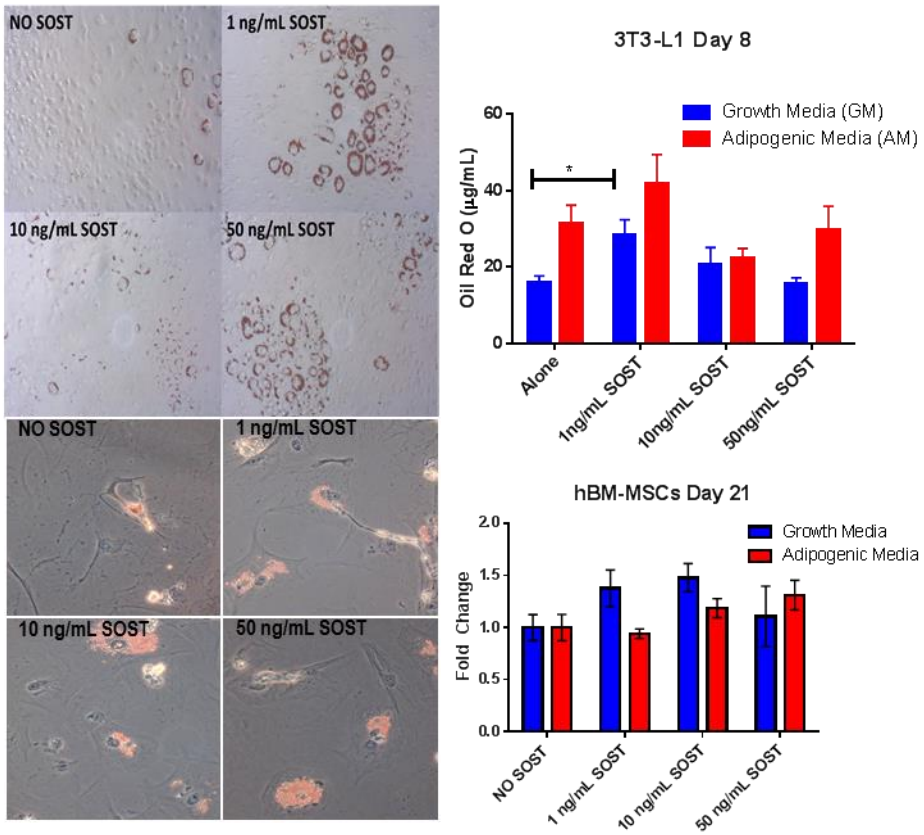

Figure 22: 3T3-L1 Cells and human bone marrow MSCs (hBM-MSCs) Increase Adipogenesis in Response to Sclerostin. Oil-Red-O Stained Representative Images (left) and quantification of oil-red-O dissolved from cells (right) from 3T3-L1 cells (top) and hMSCs (bottom) show an adipogenic response to SOST treatment. Cells grown in adipogenic or growth media responded with increased lipid accumulation, which was significant at the 1ng/mL SOST treatments in the 3T3-L1 cells in growth media, and trending towards significance in many of the other conditions. This suggests a novel role for sclerostin in the BM microenvironment.

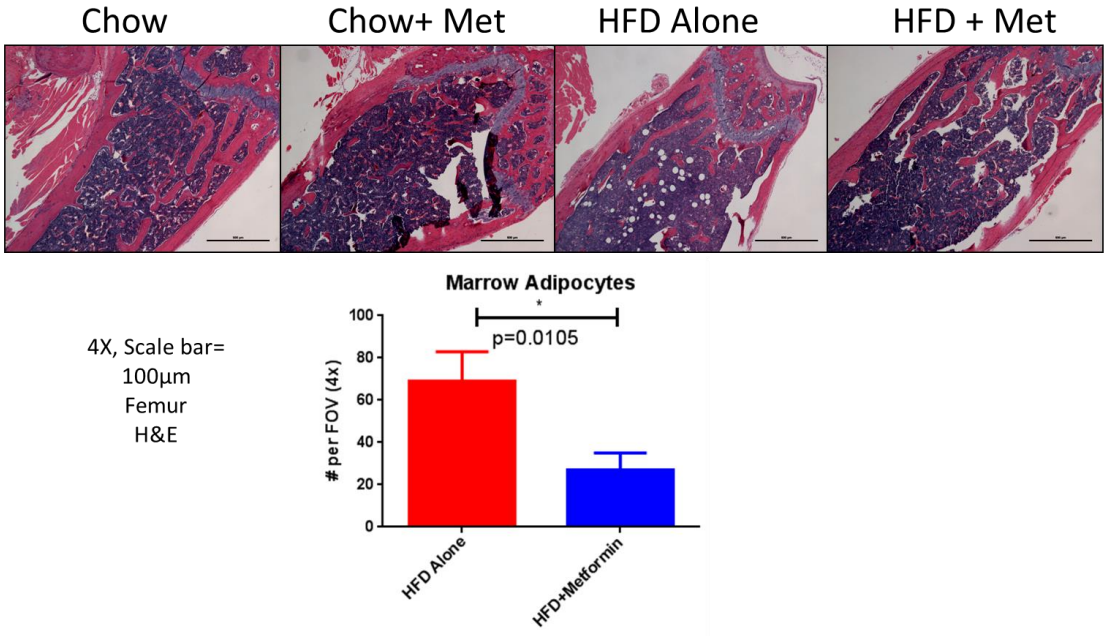

Figure 23: Metformin decreases bone marrow adipose in HFD-fed mice. Bone marrow adipose tissue (MAT) is a complex and dynamic depot that likely includes both constitutive and regulated cell populations. These representative H&E images of 16 week old mice demonstrate the fat volume in the proximal tibia medullary compartments, which was quantified using image J. Osmium high-resolution microcomputed tomography also validated the effect of metformin (not shown).

Lastly, to explore the effects of MSCs at different stages of differentiation, we cultured MM1S cells with MSCs that had been differentiated for 3 days, 1 week, 2 weeks, or 3 weeks. We used multiple donors and MM1S alone controls, and used BLI (bioluminescence) in 96 well plates using a plate reader to assess if MSCs in different stages of differentiation have different effects on MM cells. In general, it appeared that most MSCs, differentiated or not, support MM cells. However, looking at Figure 24, where the effects of differentiated MSCs on MM cells is normalized to the effects of undifferentiated MSCs on MM cells, it appears that for a number of donor MSCs, the more differentiated the cells become, the less MM supportive they are. We believe the fact that they are supportive at all may be due to the fact that hMSCs are a heterogeneous population and all the cells do not differentiate. Therefore, we are re-running this experiment with hFOBS (human fetal osteoblast cells) and MC3T3s (mouse pre-osteoblasts) as the osteoprogenitors, as these are cell lines and much more homogeneous than primary hMSCs.

Osteolysis was observed in MM1S-bearing mice (Figure 25) demonstrating osteolysis for the first time in this model and suggesting it will be useful in further pursuit of cancer-induced bone disease therapies. Lastly, our working hypothesis is shown in Figure 26, which is an overview of the work done in this project.

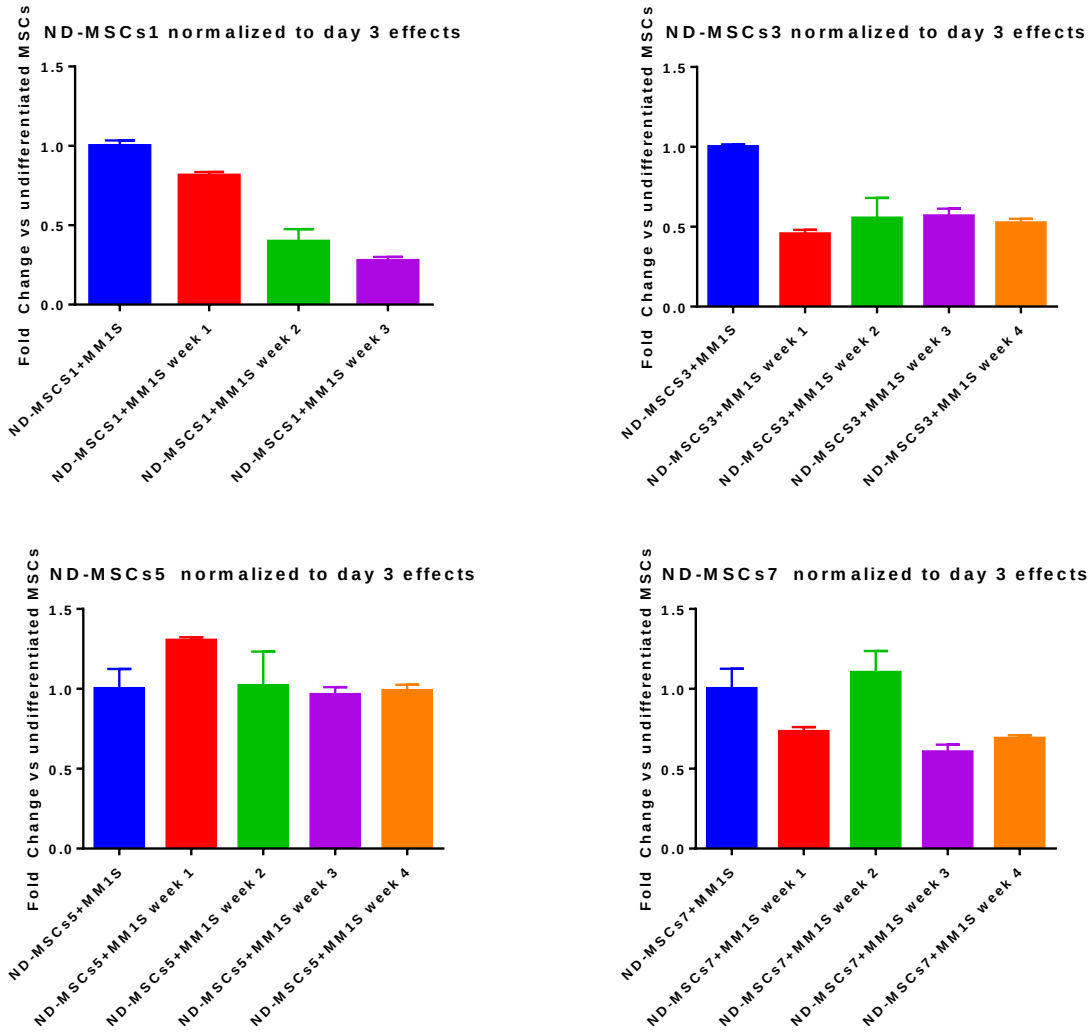

Figure 24: BLI of myeloma cells co-cultured with MSCs differentiated for different time periods normalized to effects of undifferentiated MSCs. Normal donor 1,3 and 7 were less supportive after differentiation for MM1S cells than they were when in a stem cells (MSC) state. Donor 5 did not show this decreased trend, highlighting donor heterogeneity.

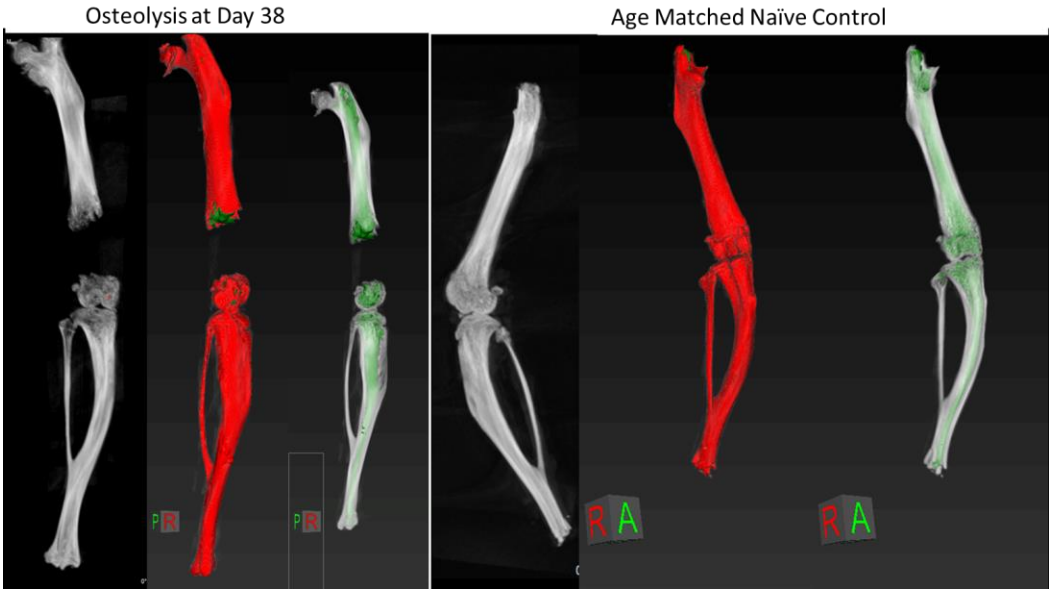

Figure 25: MM1S bearing mice have tumor burden and osteolysis throughout the bone marrow assessed by microCT. A day 38, shown here, the bone was so weak from MM1S induced bone destruction that the femur broke at the distal metaphysis. Red represented denser bone and green represented less dense bone marrow. We are further developing the tools we need to quantify bone destruction in parallel to tumor growth using uCT and BLI in parallel in the new lab.

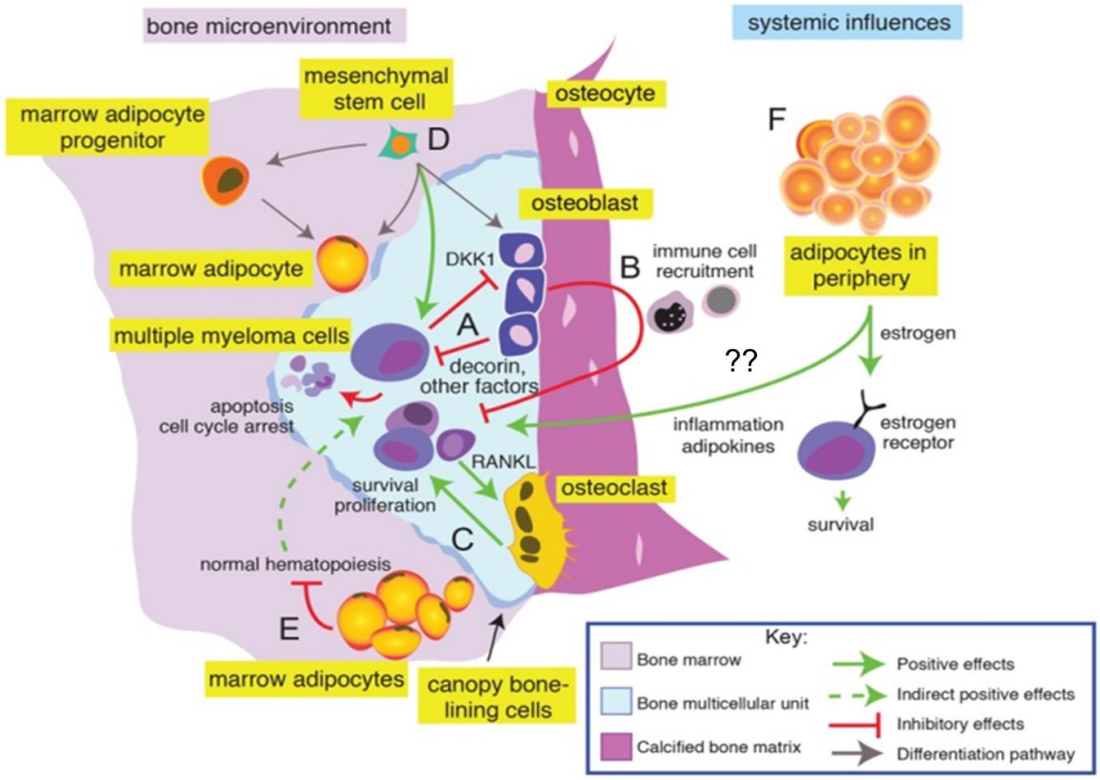

Figure 26: Complexities of the Bone Marrow Microenvironment. This working hypothesis diagram from our recent review highlights the complexities of the bone marrow microenvironment in which MM grows. Importantly, adipocytes are a relatively unexplored cell type in the bone marrow, despite the fact that they likely contribute to tumor growth in a multitude of ways. Our work here not only looked at the effects of MM on osteocytes and osteoblasts, but also began to make ties between these cells and a sister-cell in their lineage path, the bone marrow adipocyte. We realized that in our attempts to target the bone, with both our OcnCre/iDTR mouse models and anti-sclerostin antibodies, we were also having significant impact on the bone marrow adipose depot. We have now shown that this may be due to sclerostin secreted from osteocytes, which appears to have a pro-adipogenic effect on pre-adipocytes. Overall, our work provides new insight into the bone marrow niche and the complex relationships between cells there, which is pivotal for better comprehension of how tumors grow in the marrow, and how best we can target these. My new lab, among others worldwide, is exploring these concepts.

4. Key Research Accomplishments

- Developed the *in vitro* 3D Model of osteoprogenitors and multiple myeloma cells that is easily modifiable and able to be imaged, so that other types of cancer and bone cells can be investigated.
- Examined the roles of microRNAs in osteogenesis and identified some novel miRs (miR199a) to target for the inhibition of osteolysis.
- Developed *in vivo* model of inhibited osteogenesis using OcnCre;DTRmut transgenic mice, but noticed that the bone marrow of these mice was completely different from littermate controls, and further went on to explore why and how these mice had significantly increased BMAT (bone marrow adipose tissue levels), in an effort to better characterize the model before MM cells are introduced here.
- We better characterized the OcnCre/iDTR model and became more interested in better characterizing the model's BMAT phenotype first, since without a good characterization of this previously unreported phenotype, it is too complicated to elucidate the effects of different cellular components within it on the growth of MM (Vk*Myc).
- Demonstrated that Sclerostin, an osteocyte-derived factor, is able to induce adipogenic differentiation in multiple cell types. Validated this in an *in vivo* model.
- Developed model systems of 3D adipose to explore the
- We built on work described to study the potential for targeting the bone marrow, rather than the MM cells directly, to have an anti-cancer effect on multiple myeloma, as published in Blood[8], shown below.
- We developed Novel Alendronate- PEG-PLGA, Bortezomib loaded nanoparticles that we are exploring for utility in the clinic. Validated these, as published in a PNAS manuscript[7], showing efficacy of bortezomib on the bone microenvironment (increased strength, size) and subsequent inhibition of tumor burden. We showed these are non-toxic, bone-homing, and able to deliver their drug load.
- Developed a collaboration with Novartis that provided us with Anti-Sclerostin Antibodies that proved the proof-of-concept that treating mice with MM1S with a bone anabolic, anti-sclerostin antibodies (SOST-Abs) can increase bone mass, and decrease tumor burden. This work was done with our collaborators who found in the 5TGM1 mouse myeloma model, that SOST-Abs strengthen bones, increase trabecular and cortical bone volume fraction, and increase bone formation rates by osteoblasts.
- Based on our work, we also continued to explore the biology of the bone marrow microenvironment and the effects of diet (High fat diet vs low fat diet) and metformin (an anti-diabetes drug) on systemic and local (bone marrow) changes that might affect how multiple myeloma disease progresses in mice and humans. We made a number of discoveries about the bone marrow in this model that had never been observed before, most notably, that high fat diet increases bone marrow adipose and that treatment with metformin rescues this phenotype. This led to more investigations into bone-fat-myeloma interactions.
- Lastly, we developed the novel hypothesis that there may be an osteocyte derived factor that is affecting bone marrow adipose tissue. We think that factor might be sclerostin, and we have done a few experiments that showed that sclerostin does indeed induce adipogenic differentiation.
- Data produced from this grant can be found in the publications in the Appendices.

5. Conclusions

The new preclinical bone cancer model developed here has the capacity to support long-term culture and imaging for expansion of primary myeloma cells; high-throughput drug screening; vessel formation; and osteogenesis in the presence of cancer. Our 3D model uses silk protein-based scaffolds that allow for active cell attachment and adherence to scaffolds, rather than a passive encapsulation in 3D hydrogel cultures. In addition, the tissue-engineering approach represents a more controllable model compared to culturing whole patient bone biopsies[16], as it allows for user-designed introduction of cells of interest, increasing the reproducibility, adaptability, and scalability of the model. The system was used to identify miR-199a as partially responsible for decreased osteogenesis observed in myeloma patient osteoprogenitors. Although the exact composition and interaction of mRNAs inhibited by miR-199a appears complex, it is clear that miR-199a represents the first

abnormally expressed miRNAs in bone cancer patients that may be a therapeutic strategy for enhancing bone formation.

The novel nanoparticles developed here have high potential for clinical utility, and we are working now with another company to try to develop similar types of nanotubes loaded with bortezomib and coupled to alendronate into mice. We are able to do this because we are building on our work in this grant with PEG-PLGA drug filled nanoparticles. We also build on our work explore bone and cancer interactions to explore the use of anti-sclerostin antibodies and saw exciting results (stronger bone, more bone mass, decreased tumor growth in mice) that we are now further exploring and hope to publish with Dr. Croucher and Dr. McDonald, our colleagues. Lastly, with Dr. Rosen, we are building on the findings we made in the OcnCre/iDTR model and anti-sclerostin antibody treatments that sclerostin may not only target pre-osteoblasts, but that it may also target adipocytes in the bone marrow. This could have big implications in disease beyond multiple myeloma, such as osteoporosis and obesity. We plan to use this work as preliminary data for an R01 within the next year.

Over the past 2 years, this Visionary Post-Doc Fellowship has had a monumental effect on my career and has provided me with the financial security that has allowed me to: perform outstanding research, push my research interests outside my comfort zone to begin to explore unknowns in the bone biology world, participate in numerous scientific meetings and committees, publish high-impact reviewers and manuscripts, network to meet my new mentor, Dr. Clifford Rosen, who recruited me to Maine and gave me a generous start-up package for my own lab, and allowed me to pass on this gift by teaching others and spreading the word about my research and science in general, to people ranging from undergraduate students in my new lab, to international leaders in the blood cancer field. This simply would not have been possible if the research funds here were not generous to allow this to all happen, as I would have been burdened looking for a job, trying to write grants to fund my research, and I would not have had the ability to think creatively, explore new ideas, and make connections and new collaborations with researchers slightly outside the field of myeloma. Most importantly, I now have the ability to dedicate the next 5 years of my life to the study of myeloma and bone disease, my passion, and I will be able to build on the tools, skills, scientific knowledge, and understanding developed during my Fellowship.

6. Publications, Abstracts and Presentations

1. Lay Press:

1. Press Release: **Scientists Engineer Nanoparticles to Prevent Bone Cancer, Strengthen Bones** http://www.dana-farber.org/Newsroom/News-Releases/Scientists-Engineer-Nanoparticles-to-Prevent-Bone-Cancer-Strengthen-Bones.aspx. June 30, 2014.

2. DFCI's Magazine: Paths of Progress, Page 18-19. Spring/Summer 2015. See Appendix 3.

2. Peer-Reviewed Scientific Journals:

Peer-Reviewed Scientific Journals Publications:

1. Swami A & Reagan MR*, Basto P, Mishima Y, Kamaly N, Glavey S, Zhang S, Moschetta M, Seevaratnam D; Zhang Y, Liu J, Memarzadeh T, Wu J, Manier S, Shi J, Bertrand N, Lu ZN, Nagano K, Baron R, Sacco A, Roccaro AM, Farokhzad OC, Ghobrial IM. 2014. Engineered Nanomedicine for Myeloma and Bone Microenvironment Targeting. *PNAS;*111(28):10287-922014. **Co-first authorship.* Acknowledgement of federal support (yes).

2. Reagan MR, Mishima Y, Glavey S, Zhang Y, Manier S, Lu ZN, Memarzadeh M, Zhang Y, Sacco A, Aljawai Y, Tai Y-T, Ready JE, Shi J, Kaplan DL, Roccaro AM, Ghobrial IM. 2014. Investigating osteogenic differentiation in Multiple Myeloma using a novel 3D bone marrow niche model. *Blood*;124(222):3250-3259. Acknowledgement of federal support (yes).

3. Reagan MR, Liaw L, Rosen CJ, Ghobrial IM. Dynamic interplay between bone and multiple myeloma: emerging roles of the osteoblast. *Bone.* 2015; 75:161-9. PMCID: PMC4580250. Acknowledgement of federal support (yes).

4. Roccaro AM, Mishima, Y, Sacco A, Moschetta M, Shi J, Zhang Y, Reagan MR, Huynh D, Kawano Y, Sahin I, Chiarini M, Manier S, Cea M, Aljawai Y, Glavey S, Pan C, Cardarelli P, Kuhne M, Ghobrial IM**.** 2015. CXCR4 regulates extra-medullary myeloma through epithelial-mesenchymal-transition-like transcriptional activation. *Cell Rep.* Jul 28;12(4):622-35. PMID: 26190113. Acknowledgement of federal support (yes).

5. Reagan, MR and Rosen CJ. 2015 Navigating the Bone Marrow Niche: Translational Insights and Cancer-Driven Dysfunction. *Nature Reviews Rheumatology*. Nov 26. PMID:26607387. Acknowledgement of federal support (yes).

6. Moschetta M, Mishima Y, Kawano Y, Manier S, Paiva B, Palomera L, Aljawai Y, Calcinotto A, Unitt C, Sahin I, Sacco A, Glavey S, Shi J, Reagan M, Prosper F, Matteo B, Chesi M, Bergsagel L, Vacca A, Roccaro A, and Ghobrial I. 2015, in press. Targeting vasculogenesis to prevent progression in multiple myeloma" [Paper #15-LEU-1128R]. Acknowledgement of federal support (yes).

7. Veld JV, O'Donnell EK, Reagan MR,Yee AJ, Torriani M, Rosen, CJ, Bredella, MA. "Body composition predictors of progression from MGUS to multiple myeloma". Under review. *European Radiology*.

8. Abbott RD, Zieba A, Marra KG, Wang RY, Rubin JP, Reagan MR, Ghobrial IM, Borowsky FE, Kaplan DL. Mature, sustainable unilocular adipose 3D tissue engineered systems. Submitted to JCI.

9. Glavey SV, Huynh D, Reagan MR, Manier S, Moschetta M, Kawano Y, Roccaro AM, Ghobrial IM, Joshi L, O'Dwyer ME. The cancer glycome: carbohydrates as mediators of metastasis. Acknowledgement of federal support (yes). Blood Rev. 2015 Jul;29(4):269-79. PMID: 25636501

3. Invited Articles and Book Chaptes

1. Fairfield H, Falank C, Avery L & Reagan MR. Multiple Myeloma in the Marrow: Pathogenesis, Models and Treatments. Revisions submitted. *Marrow-Annals of the New York Academy of Sciences*. Acknowledgement of federal support (yes).

2. Reagan, MR, McDonald, MM, Croucher, P. Fat, Bone and Myeloma; Progression or Regression. Special Issue "Fat and Bone" in *Calcified Tissue International and Musculoskeletal Research*". In progress.

3. Dadwal, U, Fairfield H, Falank C, Sterling J, and Reagan, MR. Tissue Engineered 3D Bone for Cancer-in-Bone Modeling. *BoneKEy Reports*. In progress.

4. Falank C, Fairfield H, Fan Y, Tirrell S, Lanske B, Rosen C, Reagan M. Bone marrow adipose tissue: formation, function, and impact on health and disease. *Frontiers in Endocrinology.* In progress.

5. Chapter "Causes of Cancer" for the book "Cancer: Prevention, Early Detection, Treatment and Recovery", edited by Dr. Gary Stein, UVM. Submitted.

4. Abstracts

Oral Presentations/Abstracts:

Anti-Sclerostin Treatment Prevents Multiple Myeloma Induced Bone Loss and Reduces Tumor Burden. Michaela R Reagan, Michelle McDonald, Rachael Terry, Jessica Pettitt, Lawrence Le, Sindhu Mohanty, Ina Kramer, Michaela Kneissel, Daniel J Brooks, Mary Bouxsein, Clifford Rosen, Irene Ghobrial, Peter Croucher.

Oral Presentation and Abstract Achievement Award, American Society of Hematology (ASH) Annual Meeting, December 2015, Orlando, Florida.

Metformin Increases Bone Mass, Reduces Adipocyte Size and Significantly Changes Circulating Metabolomics in B6 mice Only During States of Energy Excess. Aug 2015. Sun Valley Workshop, ID.

Nanoparticles for Bone-Specific Chemotherapy and Microenvironmental Targeting in Myeloma. Post-Doctoral Retreat September 18, 2014. Boston, MA, USA.

Invited Speaker and Honorarium Recipient, May 2014. Center for Clinical & Translational Research, Maine Medical Center Research Institute

Modeling Multiple Myeloma in a Tissue-Engineered Bone Marrow Niche. IBMS: Herbert Fleisch Workshop. March, 2014. Brugge, Belgium.

Nanoparticle Design For Bone-Specific Chemotherapy and Microenvironmental Targeting In Multiple Myeloma. ASH Conference Dec. 10, 2013. New Orleans, Louisiana.

Multiple Myeloma bone marrow derived mesenchymal stem cells (MSCs) show decreased osteogenesis in part due to decreased expression of microRNA hsa-mir-199a-3p, miR-15a-5p and miR-16-5p. IBMS- Cancer and Bone Society Conference, November 7-10, 2013. Miami, Florida.

Poster Presentations/Abstracts (First Authorship):

Metformin Increases Bone Mass, Reduces Adipocyte Size and Significantly Changes Circulating Metabolomics in B6 mice Only During States of Energy Excess. American Society of Bone and Mineral Research (ASBMR) Annual Meeting October 2015, Seattle WA.

Metformin Increases Bone Mass, Reduces Adipocyte Size and Significantly Changes Circulating Metabolomics in B6 mice Only During States of Energy Excess. ASBMR Meeting Sept 2015, Bar Harbor, ME.

Bone-Specific Nanoparticle Delivery for Microenvironmental Targeting in Multiple Myeloma. Skeletal Research Center Annual Symposium, MGH. June, 2015. Boston, MA.

Nanomedicine Design for Bone Microenvironment Targeting in Multiple Myeloma. ECTS/IBMS Meeting. April 2015. Rotterdam, Netherlands.

Modeling Multiple Myeloma in a Tissue-Engineered Bone Marrow Niche. IBMS: Herbert Fleisch Workshop. March, 2014. Brugge, Belgium.

MicroRNA-Dependent Modulation Of Osteogenesis in a 3D In Vitro Bone Marrow Model System Of Multiple Myeloma. ASH Conference Dec. 8, 2013. New Orleans, Louisiana.

Multiple Myeloma bone marrow derived mesenchymal stem cells (MSCs) show decreased osteogenesis in part due to decreased expression of microRNA hsa-mir-199a-3p, miR-15a-5p and miR-16-5p. Presented at IBMS-CIBD Conference, November 7-10th 2013. Miami, Florida.

Novel Target Identification: Multiple Myeloma bone marrow derived mesenchymal stem cells (MSCs) how decreased osteogenesis in part due to decreased expression of microRNA hsa-mir-199a-3p. Dana-Farber Cancer Institute Post-Doctoral Retreat, September 20, 2013. Cambridge, Massachusetts.

7. Inventions, Patents and Licenses

No patents or licenses to report

8. Reportable Outcomes

We developed a 3D bone marrow tissue engineered platform that we used to investigate the way that multiple myeloma grows in the bone marrow and how it impairs osteogenesis. We have built on this model and now it is being used in our lab for other applications, for example, for studying how osteocytes and myeloma cells grow together in a 3D environment.

The 3D model culture technique has been published and at the request of researchers at the Garvan Institute in Sydney, Australia, samples of silk scaffolds have been sent to Dr. Michelle McDonald and Dr. Peter Croucher there. We will help them use these scaffolds to model dormancy of myeloma cells in the bone marrow niche and are committed to helping any other researcher who would like to try to use the model by sending some sample scaffolds or teaching them to make their own and helping them troubleshoot any issues.

We have also developed novel bone marrow-targeting PEG-PLGA alendronate-coated, bortezomib-loaded nanoparticles that we may be used, eventually, for patients with metastatic bone disease or multiple myeloma.

We also developed the use of anti-sclerostin antibodies (SOST-Abs) to treat multiple myeloma in the MM1S SCID-beige in vivo model of myeloma and found that it was successful in inhibiting tumor growth and strengthen bones, suggesting that increasing bone density could be useful in MM patients. We are in talks with Novartis and Amgen to see if we can continue these collaborations so that we can get these treatments into patients by repurposing SOST-Abs that are already in Phase III clinical trials for osteoporosis (*Romosozumab*).

We further characterized the bone marrow phenotype of our OcnCre/iDTR mice in these studies and made the observation that, although there is low bone phenotype, there is in fact a very high amount of bone marrow adipose in these models. This has led to a new way of using the OcnCre/iDTR models to investigate multiple myeloma response to adipose tissue, or investigating mechanisms driving bone marrow adipose accumulation, important for diseases such as obesity, osteoporosis, and bone healing.

9. Other Achievements

I received a highly competitive tenure-track Faculty position offer from the Maine Medical Center Research Institute for the position of Faculty Scientist I & Assistant Professor, the University of Maine.

Cell lines are being developed that have GFP and Luciferase expression in a Vk*Myc cell line. Protocols for genotyping and profiling Osteo-transgene mice will be made available once that research is complete and published, and all protocols for the 3D model have been published in *Blood*[8] and for the Bortezomib Nanoparticle work have been published in *PNAS*[7] . Data has been uploaded to the GEO database that describes the microRNA expression differences in healthy donor vs myeloma patient donor MSCs.

We have also generated some repositories of human bone marrow stem cells from both healthy donors and MM patients in this work that we have stored that we can use in future experiments.

Grants Drafted or Submitted During the Final Year of this Grant:

- Tufts CTSI pilot grant- Decision: Unknown
- NIDDK R24 entitled “Interdisciplinary Study of Marrow Adiposity, Mineral Metabolism, and Energy Balance” with new Mentor. Role; 5% effort as collaborator. Decision: Awarded
- COBRE pilot project from MMCRI- Maine Medical Center Research Institute. Decision: Awarded
- DFCI SPORE pilot project- Decision: Not awarded
- MMRF – Post-Doc Fellowship Award. Role: PI/mentor. Decision: Not funded
- NIDDK DiaComp 8 month Pilot Project. Role: PI. Decision: Score: 3.3 (1-9 scale, 1 being the best). Not funded
- COBRE- 3 year project. Role: Project PI/ Lead. Decision: Unknown (due January 2016)
- ACS (American Cancer Society) Research Scholar Grant. Role: PI. Score: Excellent, not funded. Revised Application due and will be submitted in April, 2016.

Workshops applied to and accepted into during this grant:

- AACR Basic Science Translational Medicine Workshop, November 2015.

10. Opportunities for Training and Professional Development

1. ***New Positions:***
- Application to Maine Medical Center, Portland Maine, for position as assistant professor.
- Transitioned and Established a new laboratory as an Assistant Professor with a 5 year start-up offer from Maine Medical Center Research Institute and the University of Maine. Please see: http://mmcri.org/ns/?page_id=5832
-Visiting Scientist at Maine Medical Center Research Institute and has begun collaborations with her new colleagues there. This work has also allowed her to apply for funding from the NIH through a pilot grant at MMCRI in collaboration with Dr. Clifford Rosen, who she met at a conference presenting her work supported by this funding
- Nominated as a Dana-Farber Presidential Scholar by Dr. Edward Benz, DFCI CEO and President.
- Obtained the position of Lecturer at Harvard Medical School (final/official paperwork in progress).

2. Honors, Invited Speaking Engagements, Committee Participation based on or involving this work:

- Nominated for ASBMR John Haddad Young Investigator Award from the ASBMR (decision unknown as of December 2015)
- Invited Speaker, Washington University, November 24, 2015. Radiation Oncology Department.
- Invited Speaker at UVM's Dept. of Biochemisty Seminar Series, "Multiple Myeloma and the Bone Marrow Niche" October 23, 2015.
- Invited Committee Member- ASBMR- American Society of Bone and Mineral Research
- CURE (Continuing Umbrella of Research Experiences) Mentor, Summer 2014.
- Invited Speaker, Maine Medical Center Research Institute, June 2014.
- Guest Speaker, Boston University High School Summer Education Program, Calculus July 2014.
- Scientific Committee Member of joint ECTS (European Calcified Tissue Society)- CABS (Cancer and Bone Society)-IBMS Conference in Rotterdam, Netherlands. April 2015.
- Conference Session Chair: IBMS Herbert Fleisch Workshop, Brugge, Belgium 2014 and Cancer and Bone Society Meeting, Miami, FL 2013.
- IBMS Young Investigator Committee Member (2012-2014), Committee Co-chair (2013-2015).
- Herbert Fleisch Workshop Abstract Travel Award 2014.
- Best Poster Award, Dana-Farber Cancer Institute Post-Doctoral Retreat. Cambridge, MA. 9/30/2013.
- Harold Frost Award to Sun Valley Idaho for Musculoskeletal Biology Workshop, August 2015.
- AACR Committee Member, Associate Member Council (AMC)-led Fundraising Committee, 2015-2016
- ASBMR Conference Moderator: Bone Tumors and Metastasis, Seattle 2015
- Scientific Program Committee Member, Herbert Fleisch Meeting Brugge, Belgium, Feb. 2016
- Invited Lead Guest Editor for *Stem Cells International* Special Issue on *Cancer and Stem Cells*, 2015/'16
- Invited Seminar Speaker, Easter Bush Research Consortium (EBRC), Roslin Institute, Edinburgh, Scotland. 2015
- First Prize at Poster Session, Skeletal Research Annual Symposium, MGH, Boston, MA, 2015
- Veterans Affairs (VA) Study Section Committee Member, (Fall 2015 HEMA VA Merit Review)
- Invited journal and grant reviewer: Nanomedicine, Stem Cells, Blood, Tissue Engineering, British Journal of Haematology, Annals of Hematology, Haematologica, Prostate Cancer UK, Cancer Cell International, Cell Metabolism, PLoSOne, Bone, Marrow, BoneKEy, New England Journal of Medicine, Oncotarget.
- Undergraduate Students Trained by Michaela Reagan under this project:
  - University of Waterloo Students: Lily Lu, Tina Mehmarzadeh, Priya Dhir; 2013-2014

  - UMass Boston: Ted Hilaire, as part of the CURE (Continuing Umbrella of Research Experiences) Mentor to Undergraduate Student at (Mr. Ted Hilaire), 2014
- Lab Members Interviewed and Recruited to the Reagan Lab:
  - Heather Fairfield, BS- Research Associate
  - Lindsey Avery, MS- Rotation Student
  - Dr. Carolyne Falank, PhD- Post-Doctoral Fellow
  - Sadie Tirrell- Undergraduate Intern
  - Katherine Bonawitz- Undergraduate Intern
  - Sarah Linehan- High School Student
- Extensive One-on-One Training Time with New Mentor, Dr. Clifford Rosen. New Mentoring Committee established, composed of Drs. Mary Bouxsein, Clifford Rosen, Lucy Liaw, Don St. Germain, and Vicki Rosen.

11. References

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6. Fuhler GM, Baanstra M, Chesik D, et al. Bone marrow stromal cell interaction reduces syndecan-1 expression and induces kinomic changes in myeloma cells. *Exp. Cell Res.* 2010;316(11):1816–28.
7. Swami A, Reagan MR, Basto P, et al. Engineered nanomedicine for myeloma and bone microenvironment targeting. *Proc. Natl. Acad. Sci. U. S. A.* 2014;111(28):10287–92.
8. Reagan MR, Mishima Y, Glavey S V, et al. Investigating osteogenic differentiation in multiple myeloma using a novel 3D bone marrow niche model. *Blood*. 2014;124(22):3250–9.
9. Yu VWC, Saez B, Cook C, et al. Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *J. Exp. Med.* 2015;212(5):759–74.
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13. Reagan MR, McDonald MM, Terry R, et al. Anti-Sclerostin Treatment Prevents Multiple Myeloma Induced Bone Loss and Reduces Tumor Burden (Abstract #83746). *Am. Soc. Hematol. Annu. Conf.* .

14. Chang S-H, Luo S, O'Brian KK, et al. Association between metformin use and transformation of monoclonal gammopathy of undetermined significance to multiple myeloma in U.S. veterans with diabetes mellitus: a population-based cohort study. *Lancet. Haematol.* 2015;2(1):e30–e36.

15. Zi F-M, He J-S, Li Y, et al. Metformin displays anti-myeloma activity and synergistic effect with dexamethasone in in vitro and in vivo xenograft models. *Cancer Lett.* 2015;356(2 Pt B):443–53.

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12. Appendices: Full Journal articles (2), Review Articles (2), Lay Communications (2)

I. Reagan MR, Mishima Y, Glavey S, Zhang Y, Manier S, Lu ZN, Memarzadeh M, Zhang Y, Sacco A, Aljawai Y, Tai Y-T, Ready JE, Shi J, Kaplan DL, Roccaro AM, Ghobrial IM. 2014. Investigating osteogenic differentiation in Multiple Myeloma using a novel 3D bone marrow niche model. *Blood;* 124 (22):3250-3259.

II. Swami A & Reagan MR*, Basto P, Mishima Y, Kamaly N, Glavey S, Zhang S, Moschetta M, Seevaratnam D; Zhang Y, Liu J, Memarzadeh T, Wu J, Manier S, Shi J, Bertrand N, Lu ZN, Nagano K, Baron R, Sacco A, Roccaro AM, Farokhzad OC, Ghobrial IM. 2014. Engineered Nanomedicine for Myeloma and Bone Microenvironment Targeting. *PNAS;*111(28):10287-922014. **Co-first authorship.*

III. Paths of Progress: Research and Care at the Dana-Farber Cancer Institute, Spring/Summer 2015. Pg 18-19.

IV. Reagan, MR and Rosen CJ. 2015 Navigating the Bone Marrow Niche: Translational Insights and Cancer-Driven Dysfunction. *Nature Reviews Rheumatology*. Nov 26. Epub ahread of print.

V. Reagan MR, Liaw L, Rosen CJ, Ghobrial IM. 2015. Dynamic interplay between bone and multiple myeloma: emerging roles of the osteoblast. *Bone;* 75:161-9. PMCID: PMC4580250.

VI. Inside the Institute, July 29, 2014. Page 1, Continued on page 3.

Regular Article

LYMPHOID NEOPLASIA

Investigating osteogenic differentiation in multiple myeloma using a novel 3D bone marrow niche model

Michaela R. Reagan,[1] Yuji Mishima,[1] Siobhan V. Glavey,[1] Yong Zhang,[1] Salomon Manier,[1] Zhi Ning Lu,[1] Masoumeh Memarzadeh,[1] Yu Zhang,[1] Antonio Sacco,[1] Yosra Aljawai,[1] Jiantao Shi,[2] Yu-Tzu Tai,[1] John E. Ready,[1,3] David L. Kaplan,[4] Aldo M. Roccaro,[1] and Irene M. Ghobrial[1]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; [2]Department of Biostatistics, Harvard School of Public Health, Boston, MA; [3]Brigham and Women's Hospital, Boston, MA; and [4]Department of Biomedical Engineering, Tufts University, Medford MA

Key Points

- 3D bone marrow niche model recapitulates in vivo interactions of tumor and bone cells in a more biologically relevant system than in 2D.
- Differential expression levels of miRs in MSCs provide novel insights into mechanisms of regulation of osteoblasts in multiple myeloma.

Clonal proliferation of plasma cells within the bone marrow (BM) affects local cells, such as mesenchymal stromal cells (MSCs), leading to osteolysis and fatality in multiple myeloma (MM). Consequently, there is an urgent need to find better mechanisms of inhibiting myeloma growth and osteolytic lesion development. To meet this need and accelerate clinical translation, better models of myeloma within the BM are required. Herein we have developed a clinically relevant, three-dimensional (3D) myeloma BM coculture model that mimics bone cell/cancer cell interactions within the bone microenvironment. The coculture model and clinical samples were used to investigate myeloma growth, osteogenesis inhibition, and myeloma-induced abnormalities in MM-MSCs. This platform demonstrated myeloma support of capillarylike assembly of endothelial cells and cell adhesion–mediated drug resistance (CAM-DR). Also, distinct normal donor (ND)- and MM-MSC miRNA (miR) signatures were identified and used to uncover osteogenic miRs of interest for osteoblast differentiation. More broadly, our 3D platform provides a simple, clinically relevant tool to model cancer growth within the bone—useful for investigating skeletal cancer biology, screening compounds, and exploring osteogenesis. Our identification and efficacy validation of novel bone anabolic miRs in MM opens more opportunities for novel approaches to cancer therapy via stromal miR modulation. (*Blood*. 2014;124(22):3250-3259)

Introduction

Increasing evidence demonstrates that matrix stiffness, geometry, chemistry, and spatial dimensionality, along with neighboring cells and soluble factors, regulate cellular behavior and tissue formation.[1] However, current in vitro multiple myeloma (MM) research is conducted on 2D in vitro culture plates, highlighting the need for more realistic 3D in vitro models of myeloma growth.[2] Many 3-dimensional (3D) culture and coculture systems have been described for MM and have validated the importance and relevancy of using 3D rather than 2D culture systems to more accurately model myeloma growth. Some of these models have used hydrogels (made from permutations of collagen, fibronectin, and Matrigel[3,4]), which are, as with our model, advantageous as simple, controllable, and reproducible 3D culture microenvironments useful for studying pharmaceuticals or biological pathways. However, our system transcends these properties to comprise a model representative of a mineralized bone microenvironment using bone marrow (BM)-derived mesenchymal stromal cells (MSCs) that are stimulated to undergo osteogenic differentiation on the strong, porous silk scaffolds, which does not occur on softer substrates. This is a critical component to a 3D model of myeloma and bone, because myeloma cells respond differently to undifferentiated MSCs compared with MSCs differentiated into osteoblasts and osteocytes.[5] On the other end of the spectrum are the models that use 100% biologically relevant patient-derived, whole-bone cores,[6] taken directly from patients, which have the advantage of providing a hard, mineralized, bony matrix but that lack the reproducibility, adaptability, scalability, controllability, and simplicity that characterize our tissue-engineered bone (TE-bone) model. Although this is beneficial for small-scale, individualized patient analyses, patient samples vary widely in results and responses in terms of myeloma growth and drug response, making large drug screens or biological pathway analyses impossible. Moreover, the 3D bioreactor system necessary for patient-bone core culture makes the system much more time- and cost-consuming than 3D TE-bone, which can be completely user-defined in terms of size, shape, porosity, and other parameters, and can be produced as hundreds of identical samples. Silk scaffolds, the platform of our TE-bone, can also be modified in terms of pore size, dimensions, Young's modulus, degradation speed, and seeded cellular components. Finally, our TE-bone can be used in vitro or in vivo, monitored using live, nondestructive optical imaging, and processed using flow cytometric

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techniques for analysis of cellular populations. Herein we use this novel disease model to demonstrate real-time inhibition of osteogenic differentiation in response to myeloma cells.

Osteolytic cancers such as MM develop via forward-feedback mechanisms with local MSCs in the BM, leading to devastating skeletal consequences (ie, pain, hypercalcemia, osteolysis, and fracture) and accelerated tumor growth.[7] MM cells insidiously overtake normal bone homeostasis to decrease osteoblastic activity and increase osteoclastic activity by altering local microenvironment cells.[8] MM patient–derived MSCs (MM-MSCs) exhibit decreased proliferation and osteogenesis and an inability to repair osteolytic damage, and they display great patient-to-patient heterogeneity in their ability to undergo differentiation and induce changes in MM cells.[8-10] The tumor BM microenvironment also supports tumor growth,[11] induces chemoresistance, and selects for tumor-initiating clones.[12] Therefore, a realistic model of the abnormal BM seen in MM patients would greatly benefit translational research scientists.

In myeloma patients, bone lesions with concomitant bone fractures and osteoporosis often persist despite bisphosphonate or bortezomib administration, tumor cell ablation, or disease remission.[13,14] This is partially explained by functional and gene expression differences between MM-MSCs and normal donor (ND)-MSCs.[8,15-18] However, mechanisms governing ineffectual MM-MSC osteogenesis remain unclear, and the roles of microRNAs (miRs) in this process are unknown. This highlights our need for stroma-specific targets and therapies, which can be identified only with more realistic 3D bone cancer models.

Our 3D in vitro BM model recapitulates interactions among tumor cells, stroma cells (MSCs), and endothelial cells, and the osteogenic process in normal and myeloma conditions. Our purpose was to examine dynamic cell-to-cell interactions between tumor cells and supportive cells, to determine the inhibitory effects of MM cells on osteogenesis and to develop a robust preclinical model to accelerate the rate of discovery and development of efficacious cancer treatments.

Methods

Study approval

Approval for these studies was obtained from the Dana-Farber Cancer Institute or Brigham and Women's Institutional Review Boards. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki.

TE-bone

Porous, aqueous 8% (wt/wt) silk fibroin scaffolds were made following the silk processing steps previously described[19] but were specifically designed with pores of 500 to 600 μm and cut into cylinders (5-mm × 3-mm height). Scaffolds were autoclaved for sterilization and soaked in media containing 10% fetal bovine serum 1 day before seeding. 1×10^6 MSCs were seeded onto scaffolds in regular MSC culture media and grown for 1 day, and then changed to osteogenic media. Osteogenic media consisted of α modified Eagle medium (αMEM) supplemented with 10% FBS, 100 U/mL penicillin, 10 μg/mL streptomycin (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 0.05 mM ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate. When cultured with MM1S (or without but used as controls for coculture studies), dexamethasone was excluded from the media.

Cell culture

The human multiple myeloma cell line MM1S was purchased from ATCC (American Type Culture Collection), engineered to express green fluorescent protein (GFP) and firefly luciferase (Luc^+/GFP^+ MM1S cells) as previously described,[20] and was cultured in 500 μg/mL geneticin (Invitrogen) for selection. OPM2 MM cells were labeled with red fluorescent protein (RFP) and firefly luciferase and provided by Dr Andrew Kung, Columbia University. Primary human BM–derived MSCs obtained from normal healthy subjects (ND-MSCs) or MM patients (MM-MSCs) were isolated and cultured as previously described[21] in expansion media of Dulbecco's modified Eagle medium (DMEM)+20% FBS and used at passages 2 to 4. Clinical samples were collected from patients or healthy donors from the Dana-Farber Cancer Institute and Brigham and Women's Hospital. Primary patient samples were isolated from MM patient BM aspirates using MACS technology (Miltenyi Biotec) with beads for CD138 as recommended by the manufacturer, and the negative fraction was seeded to flasks and grown as previously described to isolate BM-MSCs.[21] For imaging assays, MSCs were labeled with either the Celltracker dye DiD (Invitrogen) or calcein for live-cell imaging (Invitrogen), or they were stably transfected with the TurboRFP gene (Thermo Scientific) subcloned into pCW307 lentivirus vector (Addgene). Primary patient myeloma cells and MSCs were cocultured in "50-50" medium: a base of 50-50 F12-DMEM (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 10 μg/mL streptomycin (Invitrogen), and 2 mmol/L L-glutamine (Invitrogen). RFP-labeled HUVECS (RFP-HUVECS) were purchased from Angioproteomie and expanded in Endothelial Medium (EGM-2 BulletKit media; Lonza). All experiments were performed at 37°C, 5% CO_2 in normoxia.

3D scaffold coculture

Fluorescent (TurboRFP or DiD^+) MSCs were seeded onto scaffolds as described.[19] 1×10^6 MSCs were seeded in MSC growth media 1 day before seeding with 1×10^6 GFP^+MM1S cells. GFP^+MM1S cells alone, MSCs alone, or cocultures of $MSCs^+$ GFP^+MM1S cells were cultured on 3D silk scaffolds in dexamethasone-free osteogenic media for the duration of the coculture experiments. Cells were monitored using confocal microscopy and isolated using fluorescent-activated cell sorting (FACS) or were fixed for histology. Primary patient MM cells were labeled with the lipophilic CellTracker dye (DiI, Invitrogen) and seeded onto scaffolds (0.5×10^6/scaffold) that had been preseeded (1 day prior) with MSCs (1×10^6) labeled with a different cell-tracker dye (DiD; Invitrogen), and cultured at 37°C, 5% CO_2. For endothelial cocultures, RFP-HUVECs were cultured with or without GFP^+MM1S cells in HUVEC media and imaged using confocal microscopy over 1 month.

Drug resistance

For 3D assays, scaffolds with MSCs, GFP^+MM1S cells, or cocultures were cultured on scaffolds in 50-50 media with or without 5 nM bortezomib (Selleck). Bortezomib was diluted in dimethylsulfoxide and stored at −20°C until use, and was then diluted in culture medium immediately before use. Scaffolds were seeded with 0.5×10^6 MSCs per scaffold and 0.5×10^6 GFP^+Luc^+MM1S cells per scaffold the following day, and 50-50 media with or without bortezomib was added immediately before use and changed twice per week. For 2D assays, 0.5×10^4 GFP^+Luc^+ MM1S cells were seeded into 96-well plates with or without a confluent layer of ND-MSCs and cultured with or without media containing 5 nM bortezomib. Cells were quantified using bioluminescence imaging (BLI) and imaged with confocal microscopy.

Fluorescent microscopy

For imaging of MSCs on scaffolds, cells were labeled for live-dead staining with calcein or the LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen) following the manufacturer's instructions. For cocultures of GFP^+MM1S and MSCs, scaffolds were nondestructively imaged weekly, using 24-well glass-bottomed dishes (1.5 mm; MatTek) with a Leica SP5X Laser Scanning Confocal Microscope using Leica LAS acquisition software. Scaffolds were imaged with 10× dry, 20× water immersion, or 63X Plan Apo objectives using 488 nm Argon, 405 nm UV diode, or white light lasers (470-670 nm). Photomultiplier tubes collected fluorescence signal from autofluorescent scaffolds (405 nm/420-440 nm), GFP^+MM1S (488 nm/500-520 nm), calcein (493/509-525), DiI (552/563-573 nm), DiD (647/660-685nm), DiR (750/775-825), TurboRFP-MSCs (553/564-616 nm), and RFP-HUVECs (555/576-619 nm), which were given pseudocolors as described. Z-stack

images were acquired and processed using LeicaLite or LeicaLAS software to create single maximum projection 3D-like images or videos. Non-confocal fluorescent microscopy was performed using an Olympus CKX41 microscope with appropriate filter cubes and an Olympus DP72 Camera and dry ×10 or ×20 objectives.

Bioluminescence imaging quantification

GFP^+Luc^+MM1S cells or scaffolds seeded with GFP^+Luc^+MM1S cells were measured for bioluminescent signal after placement into opaque, white 96-well plates with 100 μL of media and 5 μL sterile firefly D-luciferin (7.5 mg/mL) (Caliper). After incubation for 5 minutes at 37°C, the signal from MM1S cells was measured on a FLUOstar Optima plate reader.

Histology, immunohistochemistry, and alizarin red staining

Scaffolds were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, paraffin-embedded, sectioned onto glass slides, and stained with alizarin red, hematoxylin and eosin (H&E), or anti-human CD138 immunohistochemistry (primary antibody,#M7228; Dako) by the Dana-Farber Cancer Institute Specialized Histopathology Core. Immunohistochemistry was run at a 1:50 dilution and stained on Leica's Bond-III autostainer using a Leica Bond Polymer Refine Detection kit. Slides were antigen-retrieved using Epitope Retrieval I (Leica) for 30 minutes. For alizarin red staining, MSCs differentiated in osteogenic media (or osteogenic dexamethasone-free media in studies with GFP^+MM1S) were fixed for 15 minutes in 1% formaldehyde, rinsed with water, stained with Alizarin Red Solution (Sigma-Aldrich) (2% wt/vol, 4.2 pH) for 10 minutes, rinsed 3 times with water, and then imaged. Staining was quantified by dissolving Alizarin Red Solution stain from wells in 6-well plates in 1 mL of decalcification solution (Cal-EX Decalcifier; Fisher Scientific) and reading absorbance of the solution at 405 nm (200 μL per well, 96-well plates in a FLUOstar Optima plate reader).

Scanning electron microscopy and micro–computed tomography

Scanning electron microscopy images of scaffolds were taken on a Nikon Eclipse 80i microscope with a DSFi1 Nikon Color Camera with NIS Elements AR Software. Scanning electron microscopy was done using a JEOL scanning electron microscope with gold sputter coating on scaffolds after fixation in 4% PFA. Microcomputed tomography (μCT) imaging was performed on scaffolds fixed overnight in 4% PFA and transferred to 70% ethanol in 1.5 mL Eppendorf tubes on a Siemens Inveon multimodality machine (positron emission tomography–single-photon emission tomography–μCT) at the Dana-Farber Cancer Institute Lurie Imaging Facility Core.

Cell counting

MM-MSCs (n = 4 donors) and ND-MSCs (n = 4 donors) were seeded to 12-well plates (5000 stromal cells/cm^2) with or without GFP^+MM1S (1250 MM1S cells/cm^2) and cultured for 9 days in 50-50 culture media. Cells were then fixed and stained with a 10% neutral-buffered formalin, 1 μg/mL Hoechst (Invitrogen) solution for 10 minutes, and photographed (at least 3 representative fields of view/well) using brightfield and fluorescent (UV filter) microscopy with a Nikon Eclipse 80i microscope (20×), a DSFi1 Nikon Color Camera, and an NIS Elements AR Software, and then counted by a blinded investigator using the ImageJ Cell Counter plugin (v1.47). The mean number of cells/cm^2 ± standard error of the mean (SEM) was calculated and graphed.

Matrigel and fibrin-hydrogel culture

Fibrinogen (Sigma-Aldrich) and thrombin (human BioUltra recombinant, Sigma-Aldrich) were mixed to create 4-mg/mL fibrin hydrogels. Matrigel (BD Biosciences) was diluted 1:3 in phosphate-buffered saline. Both hydrogels were immediately mixed with cells (cell tracker dye DiR^+MSCs, RFP^+HUVECs, GFP^+MM1S cells, or a combination) before seeding into 96-well plates (20 000 cells/well in 100 μL) and were cultured and imaged with fluorescent confocal microscopy over 12 days.

mRNA and miR isolation and qRT-PCR

miRNAs and mRNAs were isolated from cells using the miRNeasy isolation mini-kit (Qiagen), quantified, and tested for quality and contamination using a Nanodrop machine (ThermoScientific),[21] and then subjected to quality control minimum standards of 260/230 > 2 and 260/280 > 1.8 before further use for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) or nanoString analysis. For qRT-PCR, sample mRNA was reverse-transcribed into cDNA for either miR using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) or for mRNA using SuperScript III First-String SuperMix (Invitrogen), according to the manufacturer's instructions. qRT-PCR was performed using SYBR Master Mix (SA Bioscience). Analysis was done using the $2^{-\Delta\Delta Ct}$ method, normalized to RNU6B (miR) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (mRNA). Primers were designed using the method at http://primerdepot.nci.nih.gov (2013), shown in supplemental Table 1 available on the *Blood* Web site. miR stem-loop sequences were defined using miRBase Sequence Database Release 20 (http://www.mirbase.org). Experiments were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) and consisted of an initial denaturation step of 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Data were analyzed with StepOne Software v2.1 (Applied Biosystems), which provides a threshold cycle value that was considered as the cycle in which the fluorescence begins to be distinguished from the background. All PCR reactions were run on an Applied Biosystems AB7500 Real Time PCR system using technical triplicates and were plotted as means of at least 3 different donors ± SEM.

mRNA and miR nanoString profiling

For miR and mRNA profiling, MSC mRNA (from normal or myeloma donors) or miRNA (from normal or myeloma donors or from MSCs after 2 weeks of culture in the 3D model alone or with GFP^+MM1S) was analyzed using the nanoString platform. Expression levels from the nanoString cancer gene reference code set (nCounter GX Human Cancer Reference Kit) containing 230 cancer-associated mRNAs were compared between ND-MSCs and MM-MSCs (5 donors each) normalized to the average of 6 housekeeping genes (*PGK1*, *TUBB*, *CLTC*, *GAPDH*, *GUSB*, and *HPRT1*), graphed as heat maps, and analyzed using dChip software (DNA-Chip Analyzer; Cheng Li and Wing Wong Labs, http://www.hsph.harvard.edu/cli/complab/dchip/ [2013]; >1.5-fold change (fc) and $P < .05$ required for significance). For miRNA expression analysis of the 3D model system, 5 normal stroma cultured alone or in coculture with MM1S myeloma cells, isolated by FACS after 2 weeks of coculture, were analyzed and compared with clinical samples, (3 ND-MSC and 7 MM-MSC samples analyzed at passage 2). Primary samples (normal donor or myeloma patient) and 3D model samples (coculture vs alone) were both analyzed for stromal cell expression of 800 miRNAs using the nanoString miR analysis platform (nCounter human miRNA Expression Assay) following the manufacturer's protocol. Briefly, default settings for quality control on miR samples were used to assure high-quality miR and accuracy of the experimental process for 4 parameters (imaging, binding density, positive control linearity, and control limit of detection). A total of 100 ng of mRNA and miR was used as input into the sample preparation reaction for the nanoString nCounter assay. The miRNAs were all then normalized to the top 100 miRs per sample (by averaging the expression of the top 100 miR per sample, and then dividing all miR by this number), filtered for miRs with an average of >25 counts (cutoff in nanoString units of expression to establish real expression) and considered significant for $P < .05$ using dChip software analysis, following the manufacturer's instructions and previously reported literature.[22] An abundance of specific target molecules was quantified robotically on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules on the sample cartridge with a charge-coupled device camera as reported previously.[22] For each assay, a high-density scan setting encompassing 600 fields of view was used. miRs reaching a minimum threshold of 25 counts, fc of >1.5, and significance of $P < .05$ were identified as significant and were further investigated in miR mimic assays.

miR transfection

MM-MSCs were transfected with miR-199a-3p and miR-199a-5p miR *mir*Vana mimics (Ambion) and a negative control (*mir*Vana negative control mimic #1) or miRCURY (Exiqon) inhibitors for miR-181a-5p, miR181c-5p, miR-222-3p, miR-601, miR-146a-5p, and miRCURY negative control, following the manufacturer's instructions. MSCs were cultured until they were 80% confluent and were then transfected with a final concentration of 30 nM of each miR mimic or 50 nM of each miRCURY Inhibitor for 24 hours using Lipofectamine 2000 (Invitrogen). Alizarin Red Solution staining and qRT-PCR were performed on samples after 10 days of culture in osteogenic (dexamethasone-free) media. Efficiency of transfection was validated by qRT-PCR for detection of miR levels at 24 hours and 10 days as previously described.[21]

Pathway enrichment analysis

The targets of miR-199a-5p were predicted by TargetScan[23] and retrieved from online (http://www.targetscan.org/). We used pathways derived from 3 databases—BioCarta, KEGG, and Reactome—which were downloaded from MSigDB.[24] Hypergeometric testing was used to assess the enrichment of pathways. The enrichment *P* values were adjusted to account for multiple testing, resulting in a false discovery rate for each pathway,[25] and pathways were identified using a false discovery rate cutoff of 10%.

Statistics

Statistical analysis was performed using GraphPad/Prism Version 6.02 or Microsoft Excel. *P* values are based on Student's *t* tests (2-tailed) for 2-way comparisons, or analysis of variance (ANOVA) for multiple hypothesis testing using post hoc Dunnett (2-way ANOVA) or Fisher least significant difference (LSD) (1-way ANOVA) multiple comparison testing. Sample variance was determined using an F-Test, and normality was determined using a Normal Quantile Plot to test for non-normality (Q–Q probability plot). $P < .05$ was considered significant and *P* values are provided in the figures or their captions. Statistics for heat maps were done using dChip software using $P < .05$ as significant.

Results

We first developed our TE-bone model and characterized its unique ability to represent a mineralized 3D bone matrix, not afforded by any previously described model. Silk scaffolds, which recapitulate the high-compressive strength and porous nature of the BM trabecula,[26] were seeded with ND-MSCs and differentiated into TE-bone using osteogenic media for 50 days. The TE-bone samples formed dense, calcified tissue, as demonstrated by μCT imaging (supplemental Figure 1A), scanning electron microscopy (supplemental Figure 1B), and nondestructive fluorescence confocal microscopy (Figure 1A). This 3D mineralized model served as a basis to begin studying osteogenesis in the context of myeloma.

Next we tested the ability of myeloma cells to grow in osteogenic media, which would be required in coculture. However, we found dexamethasone to be toxic to both MM1S and OPM2 cells (data not shown) and hence adapted the osteogenic media for our purposes by removing dexamethasone, an alteration that has been previously described.[27] We next tested the ability for OPM2 and MM1S cell lines to grow on the silk scaffolds and found that, although both cell types were able to adhere to ND-MSCs seeded on scaffolds, only MM1S could adhere to the silk scaffolds alone, as observed in confocal imaging (data not shown). Hence for subsequent studies we chose to use MM1S as the main myeloma cell line for this model.

We hypothesized that silk scaffolds would provide a more realistic platform to investigate endothelial cell–myeloma cell interactions in the bone marrow, so we compared coculture responses of these cells in 3D silk scaffolds and hydrogels. We cocultured fluorescent endothelial cells (RFP^+HUVECs) with GFP^+MM1S cells and observed cell-to-cell contact and interaction, as well as interesting assembly patterns unique to silk scaffold culture (Figure 1B, supplemental Figure 2A, and supplemental Video 1). Samples were imaged with confocal microscopy over 18 days and demonstrated GFP^+MM1S cell adherence to RFP^+HUVECs and incorporation into the capillary-like HUVEC structures. Myeloma cells also appeared to support the branching, tube-shaped formations of HUVECs (with observable lumens), which were not observed in HUVEC monocultures. GFP^+MM1S cells clumped and colocalized at endothelial protrusions, perhaps mimicking some of the signaling and evolution of angiogenesis within bone tumors. Interestingly, GFP^+MM1S cell association with endothelial cells in a tubelike formation may model the early stages of myeloma cell intravasation and extravasation, as well as contributions toward angiogenesis. None of these phenomena were observed in 3D fibrin hydrogel or Matrigel cultures (supplemental Figure 2B), supporting validation that the stiffer scaffold substrate more accurately recapitulates the in vivo conditions than do softer substrates.

The 3D silk scaffold model also recapitulated the ability for MSCs to protect GFP^+MM1S cells from therapeutic agents such as bortezomib over a 30-day treatment period, as quantified with bioluminescent imaging (supplemental Figure 3A) and imaged with confocal microscopy (Figure 1C). This was not achieved in 2D culture (supplemental Figure 3B), defining the 3D system as a unique environment suitable for long-term drug studies. Similarly, in vitro growth of primary MM tumor cells in 2D lacks the realistic complexity of a 3D milieu, explaining why primary patient MM cell growth was observed on MSC-seeded scaffolds over 11 days but was not possible under the same conditions in 2D culture.[28] Primary MM cells were labeled with cell-tracker dyes and imaged with confocal microscopy using calcein to assess cell viability (Figure 1D and supplemental Figures 4 and 5). They were further identified with H&E and human-CD138 stains on fixed scaffold samples to ensure plasma cell identity (supplemental Figure 6). Together, these findings indicate that our 3D BM model allows for cancer-bone modeling in a more biologically relevant system than does 2D culture or soft 3D culture.

To address the study of myeloma-induced osteogenesis inhibition, we first confirmed prior reports[8] that proliferation and osteogenesis are significantly inhibited by myeloma in clinical samples (MM-MSCs vs ND-MSCs) and in vitro 2D cocultures of ND-MSCs and myeloma cells (supplemental Figures 7 and 8). We also assessed the mRNA profile of clinical samples (ND-MSCs and MM-MSCs) by analyzing 230 mRNAs involved in cell proliferation, differentiation, migration, and other vital signaling. Unsupervised analysis demonstrated distinct clustering between ND-MSCs and MM-MSCs, confirming inherent differences between normal and myeloma stroma (supplemental Figure 9A). Forty-nine mRNAs were found to have significantly different expression between ND-MSCs and MM-MSCs ($P < .05$, 1.5-fc; supplemental Figure 9B), including the cell-cycle regulators CDKN2A (p16, previously reported[29]) and CDKN1A (p21, not previously reported), which may contribute to the decreased MM-MSC proliferation, and Collage1A1, likely contributing to decreased bone matrix formation.

We then attempted to model inhibited osteogenesis of MSCs in our 3D BM model. ND-MSCs and GFP^+MM1S were cultured alone or together on scaffolds in osteogenic media over 5 weeks. Confocal and fluorescent microscopy showed that GFP^+MM1S cells inhibited ND-MSC proliferation, migration, and tissue production in scaffolds (Figure 2A-B and supplemental Videos 2 and 3). Alizarin Red Solution and H&E staining histology of scaffolds after 5 weeks

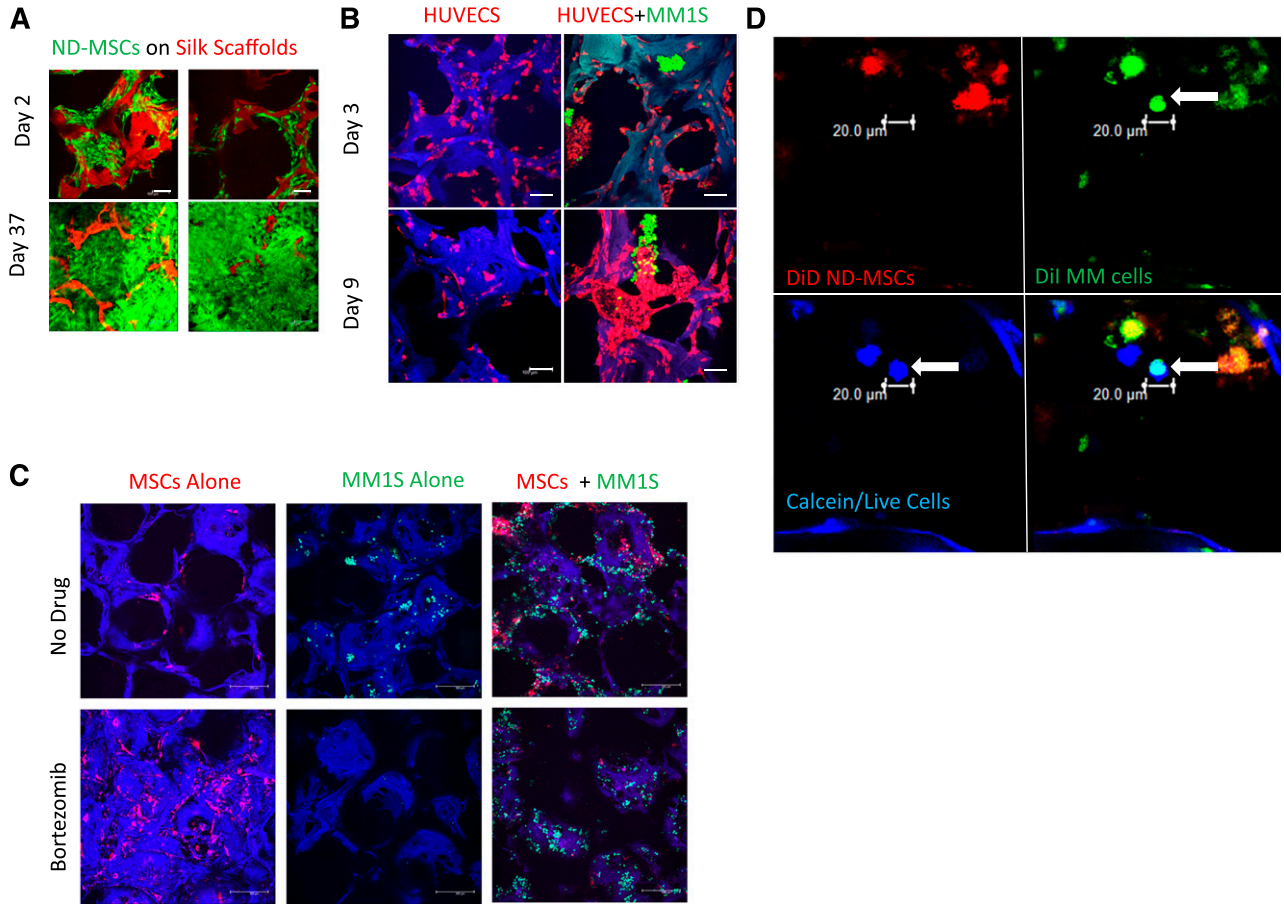


Figure 1. Development of an in vitro 3D BM niche model. (A) Confocal images of calcein-labeled ND-MSCs, passage 2, (calcein/live cells, green; silk scaffold, red) at 2 and 37 days of culture in osteogenic media. The scale bar represents 100 μm. (B) Confocal images of RFP^+HUVECs (red) ± GFP^+MM1S cells (green) on scaffolds (blue) (days 3 and 9; scale bar = 100 μm). Representative image of 3 experiments is shown here, cultured in endothelial growth media. (C) Confocal images at day 30 of culture of GFP^+MM1S alone (left, green), DiD-labeled MSCs alone (middle, red), and cocultures (right) in 50-50 medium with bortezomib (top, 5 nM) or without bortezomib (bottom) on autofluorescent scaffolds (blue). (Scale bar = 100 μm.) (D) Confocal images of primary patient $CD138^+$ MM cells (green with DiI) at day 7 seeded onto ND-MSCs (red with DiD). Channels show the myeloma cell (arrow) as alive ($calcein^+$, blue), DiI^+ (green), and DiD^- (red). Overlay of green and blue appears cyan and demonstrates colocalization of calcein and DiI staining. Samples cultured in 50-50 media (n = 3); the scale bar represents 20 μm.

demonstrated cellular tissue formation and mineralization in ND-MSC samples grown alone. Conversely, a lack of mineralization, as well as poor tissue formation and decreased cell numbers, were observed in samples of ND-MSCs cocultured with MM1S cells (Figure 2C). Interestingly, by week 2 the inhibition of MSC growth was evident in confocal imaging, and this trend continued over the full 5-week period, whereas MSCs alone proliferated, filled in scaffold pores, and formed mineralized, TE-bone. In sum, this system is useful for investigating myeloma effects on 3D osteogenesis in a more realistic setting than in 2D.

We then investigated the role of miRs in the dysfunctional osteogenesis of MSCs cultured with myeloma cells. $TurboRFP^+$ ND-MSCs were cultured alone or with GFP^+MM1S cells in the 3D model in osteogenic media for 2 weeks, sorted and collected using FACS, and analyzed for miR changes (coculture vs monoculture) using nanoString analysis of 800 miRs. Fifty-three miRs (28 up- and 25 downregulated) showed significantly altered expression in MSCs during coculture with MM1S (Figure 3A and supplemental Table 2). To compare with clinical samples, miR profiling was also performed on BM stroma samples from normal, healthy donor or myeloma patients. These samples demonstrated 41 miRs (34 up- and 7 downregulated) with significantly altered expression in MSCs from myeloma patient donors (MM) vs normal donor MSCs (ND) (Figure 3B and supplemental Table 2). Of these, six were found to be similarly downregulated (1 miR) or upregulated (5 miRs) in the 3D coculture system in the 3D model compared with the clinical samples (Table 1). The correlation between the patient and normal samples, and the BM niche 3D model contributes additional evidence that our system can reliably recapitulate many of the in vivo effects of MM cells on MSCs and suggests miRs that may govern the inhibited osteogenesis seen in patient MSCs. All miR data can be found in the GEO database under accession number GSE60423.

Finally, we examined whether any of the 6 miRs identified in the 3D model and in the clinical samples could serve as targets for inducing osteogenesis. Inhibition of miRs overexpressed in MM-MSCs (miR-181a-5p, miR181c-5p, miR-222-3p, miR-601, miR-146a-5p) using miRCURY inhibitors did not alter the mineralization potential of MM-MSCs as assessed by Alizarin Red Solution (data not shown) and hence were not further pursued. However, increasing the expression of miR-199a-5p significantly increased mineralized matrix production, indicative of osteogenic potential, and further supported the function of miR-199a as an osteogenic-promoting miR (Figure 3C-D). Using miR mimic transfection in ND-MSCs and MM-MSCs, we increased expression of miR-199a and observed that increasing the expression of both miR-199a-5p and miR-199a-3p in MM-MSCs significantly increased expression of several common osteogenic markers previously described,[30] namely, alkaline phosphatase, integrin-binding sialoprotein, collagen type I α 1, osteopontin,

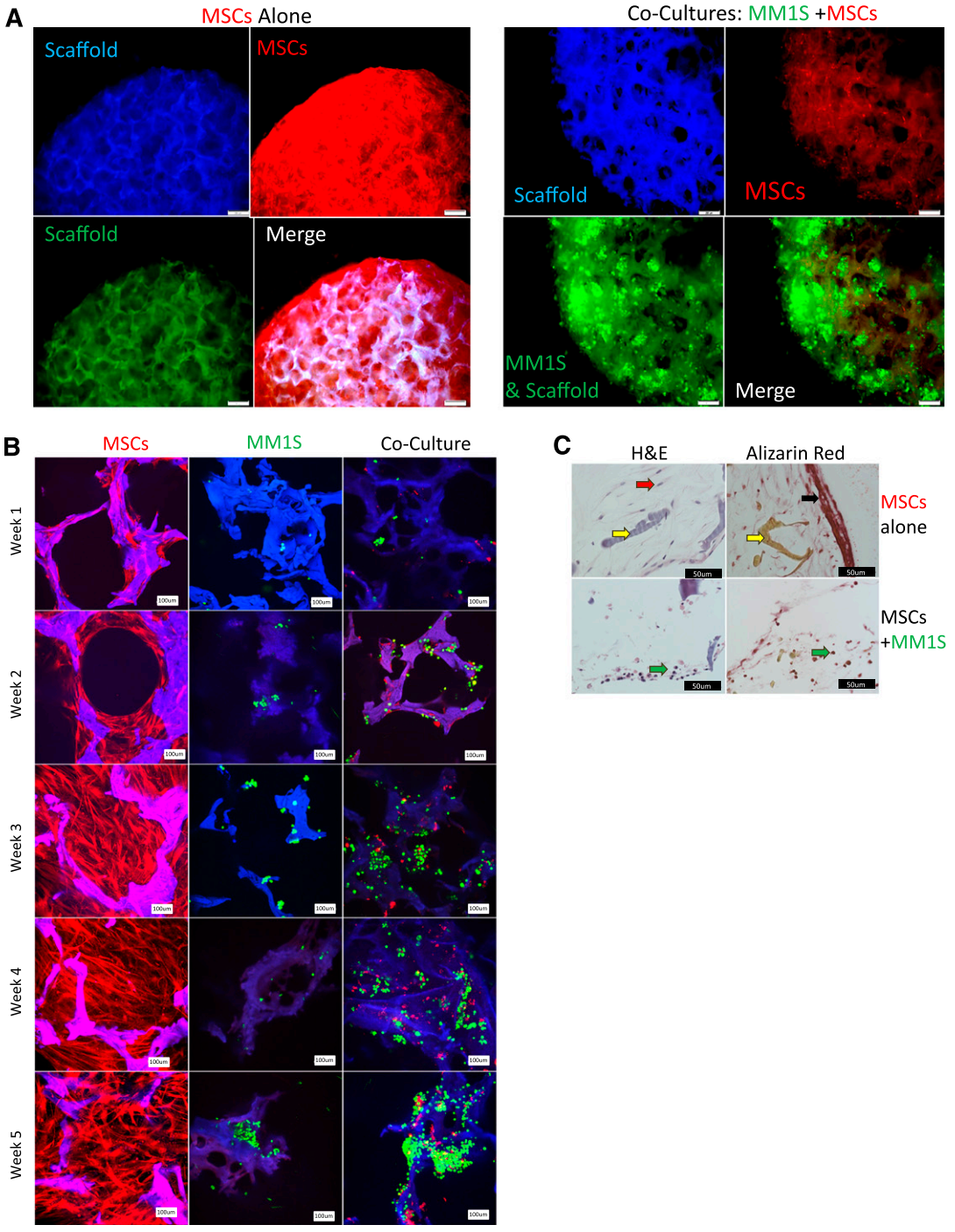


Figure 2. Inhibited osteogenesis induced by myeloma in a 3D bone model. (A) Fluorescent imaging at week 5 (TurboRFP$^+$MSCs, red; GFP$^+$MM1S, green; scaffold, blue). Overlaid channels (merge) shows increased pore infiltration, elongation, and proliferation by MSCs when grown in the absence of myeloma cells (left) compared with when grown with MM1S (right). The scale bar represents 200 μm. (B) Confocal images of TurboRFP$^+$MSCs (red) and GFP$^+$MM1S (green), alone or in coculture, on silk scaffolds (blue) from 1 to 5 weeks of culture in osteogenic media. The scale bar represents 100 μm. (C) Histologic analysis of scaffolds after 5 weeks of osteogenesis for MSCs alone (top) or in coculture with GFP$^+$MM1S (bottom) stained for mineralization (Alizarin Red, right) or hematoxylin and eosin (H&E) (left). Black arrow indicates mineralization found only in MSCs cultured alone. Yellow arrows indicate silk scaffold. Red arrow indicates stromal cells, which are found throughout the MSC alone samples and sparsely through coculture samples. Green arrows indicate MM1S plasma cells found only in coculture samples. The scale bar represents 50 μm.

osteocalcin, and runt-related transcription factor 2 (Figure 3E-F and supplemental Figure 10). Similar results were found with transfection of ND-MSCs with miR-199a-3p and 199a-5p mimics (data not shown).

To explore the potential pathways regulated by hsa-miR-199a-5p, we performed a pathway enrichment analysis of its predicted target genes. Analysis of miR-199a-5p targets revealed 19 pathways that were significantly enriched (supplemental Table 3). Interestingly, among them the ErbB signaling pathway was identified and is also reported to be involved in osteogenesis.[31,32] The MAPK signaling pathway was also identified by us, as well as by others,[33] as an miR199a-5p target pathway, and has been shown to play a role in osteogenic differentiation via the ErbB1 and ErbB2 pathways.[34] Moreover, 3 pathways centered on semaphorins were also identified

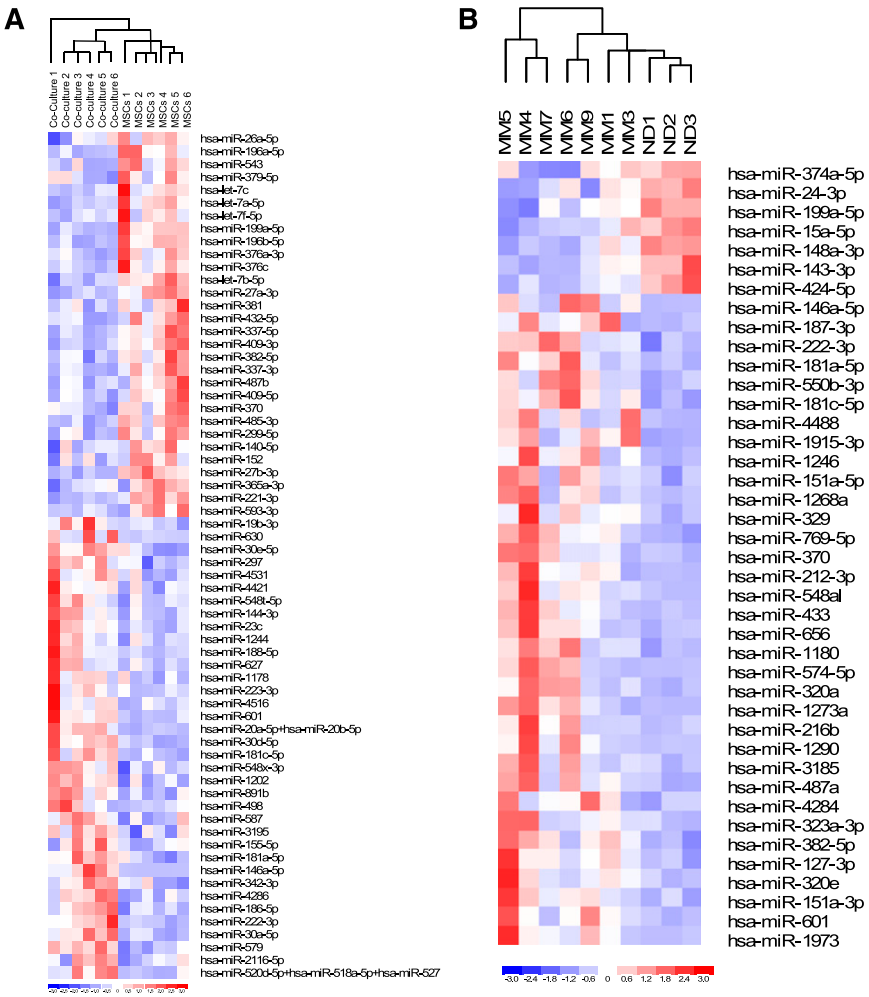


Figure 3. Alterations in MSC miRs in 3D modeling and patient vs normal data, and the resulting changes in MSCs after mimic-induced increased expression of miR-199a. (A) Heat map of the 53 mRNAs identified from nanoString analysis from the 3D model samples that are significantly different between cocultured (Co-culture) and monocultured ND-MSCs (MSCs). Filtering was done on original 800 miRs based on high expression (>25 average counts), significance between myeloma vs normal donor groups ($P < .05$), and high fc threshold (fc >1.5). (B) Heat map of 41 miRs identified from nanoString analysis that are significantly different between patient samples (myeloma [MM] and normal donor [ND] sample MSCs). Filtering was done on original 800 miRs based on high expression (>25 average counts), with significance between myeloma vs normal donor groups ($P < .05$), and high fc threshold (fc >1.5). (C) Alizarin Red staining quantification of mineralization produced by MSCs transfected with negative control mimic or miR-199a-5p mimic after 10 days in osteogenic no-dexamethasone media. Data plotted as mean ±SEM, n ≥3 different donors. (D) Alizarin Red staining representative images showing mineralization of MM-MSCs in 6-well plates transfected with negative control mimics or miR-199a-5p mimics to increase miR-199a-5p expression after 10 days in osteogenic no-dexamethasone media. Images are representative of n ≥3 different donors. The scale bars represent 200μm (original magnification ×4), 100 μm (×10), 50 μm (×20), and 20 μm (×40). (E) MM-MSCs transfected to increase expression of miR-199a-3p (E) or 199a-5p (F) demonstrate increased expression of osteogenic markers after 10 days of culture, measured by q-RT-PCR, gene expression normalized to negative control (Control) for each gene. ALPL, alkaline phosphatase; BSP, integrin-binding sialoprotein; Col1a1, collagen type I α 1; OP, osteopontin; OC, osteocalcin; RUNX2, runt-related transcription factor 2. Data plotted as mean ± SEM and analyzed with 1-way ANOVA and a post hoc Fisher least significance difference test for multiple comparisons (each gene vs negative control). Day 10 after transfection with miR mimics or controls and grown in osteogenic-no dexamethasone medium. n ≥ 3 different donors, $^{**}P < .05$, $^{**}P < .01$.

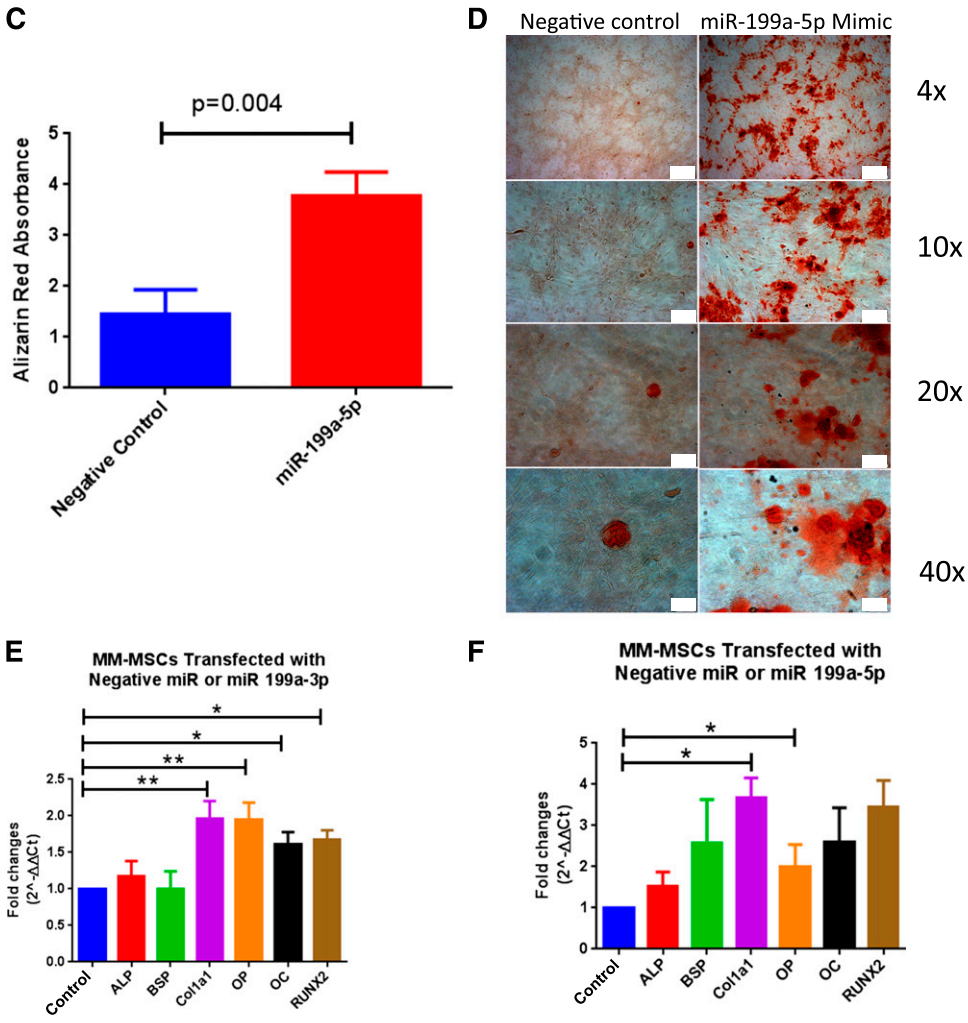
and may play a role in osteogenesis formation,[35,36] although this is currently not well-defined.[37] Hence, the pathways identified here may explain the mechanisms by which miR199a-5p regulates mRNAs that have anti-osteogenic effects (such as semaphorin4D) and may suggest novel pathways that could be targeted to normalize the osteogenic differentiation of MM-MSCs.

Discussion

3D culture models with material properties similar to those found in vivo are materializing as essential tools in cancer biology, owing to their ability to replicate tissue- or organ-specific structural features, biomechanical properties, and cell-cell or cell–extracellular matrix interactions more accurately than conventional 2D culture. Our new preclinical bone cancer model has the capacity to support long-term culture and imaging for expansion of primary myeloma cells, high-throughput drug screening, vessel formation, and osteogenesis in the presence of cancer. Prior published models have used soft, hydrogel matrices that cannot be mineralized and therefore cannot mimic the bone microenvironment.[3,4] Our 3D model uses silk protein–based scaffolds that allow for active cell attachment and adherence to scaffolds rather than passive encapsulation in 3D hydrogel cultures. In addition, the tissue-engineering approach represents a more controllable model compared with culturing whole-patient bone biopsies,[6] because it allows for user-designed introduction of cells of interest, increasing the reproducibility, adaptability, and scalability of the model. Therefore, the silk-based 3D TE-bone model presented herein represents a unique model to examine the interactions of bone and cancer cells in a 3D microenvironment, with mechanical properties similar to bone.

It remains to be determined why a decrease in certain miRs may lead to inhibited osteogenesis in myeloma and what mRNA targets drive this, but it is evident that overexpressing certain miRs within MSCs can increase their osteogenic potential, and our 3D model helped to identify 199a-5p as one such miR. MiR-199a has been described as "flexible and versatile as a chameleon,"[33] because it has a wide variety of important functions across many cell types and systems. In terms of osteogenesis, miR-199a-5p specifically has been shown to have a pro–stem cell differentiation effect in BM-derived human MSCs both in vitro and in vivo, whereas inhibition with siRNAs blocking miR-199a-5p reduced osteogenesis of hMSCs.[38,39] Pathways implicated in this are still uncertain but include HIF1a, TWIST, NADPH-oxidase, PI-3 kinase, mitogen-activated protein kinase, and NF-κB pathways, which are being investigated for their roles in osteogenesis.[38,40] miR-199a is also a BMP2-responsive miR,[41] suggesting that altered BMP2 signaling may be involved in the observed effects of these miRs. Decreased 199a-5p may also increase fibronectin in MM BM, which is elevated in MM patient

Figure 3. Continued.



serum[42,43] and has been shown to cause increased MM tumor accumulation within the BM and dictate CAM-DR.[44] Although the exact composition and interaction of mRNAs inhibited by miR-199a appear to be complex, it is clear that miR-199a represents the first miR identified as abnormally downregulated, and one of the first abnormally expressed[45] in bone cancer patients, that may be a therapeutic strategy for enhancing bone formation.

The roles of specific miRs in osteogenesis and MSC-tumor feedback are currently enigmatic,[39,46] but our results suggest that novel target miRs are useful for reactivating the osteogenic abilities of cancer-associated MSCs. Targeting these miRs may provide a new avenue for healing lesions and reversing the osteolytic cancer cycle in myeloma along with other osteotropic cancers. In conclusion, the novel 3D, in vitro bone cancer model developed provides a physiologically relevant platform to investigate osteogenesis, angiogenesis, and cancer growth, as well as drug response with primary samples and cell lines. It allows for nondestructive imaging over long periods and can be used for testing a multitude of other bone cancer hypotheses and modeling an array of biological processes involved in the inhibited osteogenesis of cancer-colonized bones. More broadly, many researchers would likely increase their in vivo success rates by first testing their hypotheses in our 3D model system. Our

Table 1. MicroRNAs altered in MSCs by myeloma

miR name	Fc, 3D model (MSCs in coculture with MM1S vs alone)	*P* value, 3D model (MSCs in coculture with MM1S vs alone)	Fc, MM vs ND MSCs	*P* value, MM vs ND MSCs
hsa-miR-199a-5p	–2.019	.00086974	–1.917	.00115665
hsa-miR-181a-5p	1.771	.00362835	3.190	.02106839
hsa-miR-181c-5p	2.135	.00276756	3.078	.00192591
hsa-miR-222-3p	2.152	.00332040	1.821	.01680615
hsa-miR-601	2.546	.00738047	3.637	.02449335
hsa-miR-146a-5p	17.175	.00405939	15.353	.02622689

Six miRs were found to be similarly upregulated (5 miRs) or downregulated (1 miRs) in the 3D system (MSCs cocultured with GFP^+MM1S vs MSCs alone, after 2 weeks in coculture in osteogenic, no-dexamethasone media) and in patient vs normal samples (MM patient MSCs vs normal donor MSCs). Fc $\geq$1.5, $P < .05$, n $\geq$3, 2-tailed Student's *t* test, average expression >25 nanoString counts.

model allows biological questions to be investigated, compounds to be screened, and novel targets or therapeutics to be identified more quickly and cheaply and in a more realistic 3D BM niche setting than is currently available. Resulting research will be more clinically translatable and will advance more quickly and efficiently from the bench to the bedside of patients who have bone cancer.

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Authorship

Contribution: M.R.R. designed the experiments, performed in vitro studies and data analysis, and wrote the manuscript; Y.M. created RFP-MSCs; YongZ., YuZ., and S.M. assisted with qRT PCR studies; J.E.R., Y.-T.T., A.S., and Y.A. provided primary MSCs or patient data; S.V.G., M.M., and Z.N.L. assisted with in vitro assays, cell culture, and qRT-PCR; J.S. performed microRNA pathway analysis; D.L.K. provided feedback and silk scaffolds; A.M.R. provided scientific assistance and advice; and I.M.G. supervised the project and edited the manuscript.

Conflict-of-interest disclosure: I.M.G. is on the advisory boards of Millennium/Takeda, Novartis, Onyx, and Celgene. All other authors declare no competing financial interests.

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Investigating osteogenic differentiation in multiple myeloma using a novel 3D bone marrow niche model

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Engineered nanomedicine for myeloma and bone microenvironment targeting

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Bone is a favorable microenvironment for tumor growth and a frequent destination for metastatic cancer cells. Targeting cancers within the bone marrow remains a crucial oncologic challenge due to issues of drug availability and microenvironment-induced resistance. Herein, we engineered bone-homing polymeric nanoparticles (NPs) for spatiotemporally controlled delivery of therapeutics to bone, which diminish off-target effects and increase local drug concentrations. The NPs consist of poly(D,L-lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), and bisphosphonate (or alendronate, a targeting ligand). The engineered NPs were formulated by blending varying ratios of the synthesized polymers: PLGA-*b*-PEG and alendronate-conjugated polymer PLGA-*b*-PEG-Ald, which ensured long circulation and targeting capabilities, respectively. The bone-binding ability of Ald-PEG-PLGA NPs was investigated by hydroxyapatite binding assays and ex vivo imaging of adherence to bone fragments. In vivo biodistribution of fluorescently labeled NPs showed higher retention, accumulation, and bone homing of targeted Ald-PEG-PLGA NPs, compared with nontargeted PEG-PLGA NPs. A library of bortezomib-loaded NPs (bone-targeted Ald-Bort-NPs and nontargeted Bort-NPs) were developed and screened for optimal physiochemical properties, drug loading, and release profiles. Ald-Bort-NPs were tested for efficacy in mouse models of multiple myeloma (MM). Results demonstrated significantly enhanced survival and decreased tumor burden in mice pretreated with Ald-Bort-NPs versus Ald-Empty-NPs (no drug) or the free drug. We also observed that bortezomib, as a pretreatment regimen, modified the bone microenvironment and enhanced bone strength and volume. Our findings suggest that NP-based anticancer therapies with bone-targeting specificity comprise a clinically relevant method of drug delivery that can inhibit tumor progression in MM.

targeting nanomedicine | alendronate-PLGA-PEG | bone metastasis | bisphosphonate

The incidence of bone metastasis is common in 60–80% of cancer patients (1). During bone metastasis, cancer cells induce a sequence of changes in the microenvironment such as secreting cytokines to increase the activity of osteoclasts via the parathyroid hormone-related protein (PTHrP), receptor activator of nuclear factor-κB ligand (RANKL), and interleukin-6 (IL-6), resulting in increased bone resorption and secretion of growth factors from the bone matrix (2). This creates a "vicious cycle" of bone metastasis, where bone marrow becomes packed with cancer cells that develop resistance to conventional chemotherapy, and leads to devastating consequences of bone fractures, pain, hypercalcaemia, and spinal cord and nerve compression syndromes (2, 3). Multiple myeloma (MM) is a plasma cell cancer that proliferates primarily in bone marrow and causes osteolytic lesions (1). Antiresorption agents, such as bisphosphonates, may alleviate bone pain, but they are ineffective at inducing bone healing or osteogenesis in MM patients (4).Bortezomib is a proteasome inhibitor that has shown marked antitumor effects in patients with MM. Proteasome inhibitors, such as bortezomib, are also effective at increasing bone formation, both preclinically and clinically (5–9). However, the major drawback of bortezomib use in early stages of MM development is its toxicity, specifically, peripheral neuropathy (5). Therefore, we aimed to develop a method to deliver bortezomib with decreased off-target side effects by using bone-specific, bortezomib-loaded nanoparticles (NPs). The NP system was based on biodegradable, biocompatible, and Food and Drug Administration (FDA)-approved components, which are both clinically and translationally relevant. NPs derived from poly(D,L-lactic-co-glycolic acid) (PLGA), a controlled release polymer system, are an excellent choice because their safety in the clinic is well established (10, 11). Polyethylene glycol (PEG)-functionalized

Significance

Limited treatment options exist for cancer within the bone, as demonstrated by the inevitable, pernicious course of metastatic breast, prostate, and blood cancers. The difficulty of eliminating bone-residing cancer necessitates novel, alternative treatments to manipulate the tumor cells and their microenvironment, with minimal off-target effects. To this end, we engineered bone-homing, stealth nanoparticles to deliver anticancer, bone-stimulatory drugs, and demonstrated their utility with bortezomib (a model drug) and multiple myeloma (a model cancer). To test our hypothesis that increasing bone volume and strength inhibits tumor growth, mice were treated with these nanoparticles before being injected with cancer cells. Results demonstrated significantly slower myeloma growth and prolonged survival. Our research demonstrates the potential of bone-homing nanomedicine as an efficacious cancer treatment mechanism.

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Conflict of interest statement: I.M.G. discloses her Advisory Board Membership with Novartis, Onyx, and BMS. O.C.F. discloses his financial interest in BIND Therapeutics, Selecta Biosciences, and Blend Therapeutics, three biotechnology companies developing nanoparticle technologies for medical applications. BIND, Selecta, and Blend did not support the aforementioned research, and currently these companies have no rights to any technology or intellectual property developed as part of this research.

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PLGA NPs are especially desirable as PEGylated polymeric NPs have significantly reduced systemic clearance compared with similar particles without PEG (12, 13). A number of FDA-approved drugs in clinical practice use PEG for improved pharmaceutical properties such as enhanced circulation in vivo (12, 13). To target NPs to bone [rich in the mineral hydroxyapatite (HA)], the calcium ion-chelating molecules of bisphosphonates represent a promising class of ligands (14). Bisphosphonates, upon systemic administration, are found to deposit in bone tissue, preferentially at the high bone turnover sites, such as the metastatic bone lesions, with minimal nonspecific accumulation (14) and were used herein to deliver NPs to the bone.

A few systems explored for MM treatment have been tested in vitro including the following: (*i*) snake venom and silica NPs (15); (*ii*) thymoquinone and PLGA-based particles (16); (*iii*) curcumin and poly(oxyethylene) cholesteryl ether (PEG-Chol) NPs (17), polyethylenimine-based NPs for RNAi in MM (18), paclitaxel-Fe_3O_4 NPs (19), and liposomes (20). However, none of the above-mentioned systems have aimed to manipulate the bone marrow microenvironment rather than the myeloma cells directly (21). To date, there are no reports of using bone-targeted, controlled release, polymeric NPs with stealth properties for MM therapy. In this study, we designed NPs bearing three main components: (*i*) a targeting element that can selectively bind to bone mineral; (*ii*) a layer of stealth (PEG) to minimize immune recognition and enhance circulation; and (*iii*) a biodegradable polymeric material, forming an inner core, that can deliver therapeutics and/or diagnostics in a controlled manner. In this study, the physicochemical properties of a range of NPs was investigated (including NP size, charge, targeting ligand density, drug loading, and drug release kinetics) and an optimal formulation with ideal properties and maximal drug encapsulation was used for in vivo efficacy studies. We fine-tuned the NP targeting ligand density to optimize its bone-binding ability and further investigated its application for targeting myeloma in the bone microenvironment. We believe our NP system has the potential to increase drug availability by improving pharmacokinetics and biodistribution that can provide bone microenvironment specificity, which may increase the therapeutic window and most certainly decrease the off-target effects (12, 13).

Results and Discussion

Design, Synthesis, and Characterization of Alendronate-PEG-PLGA NPs. The design and synthesis of alendronate-PEG-PLGA (Ald-PP), bone-targeted NPs engineered with fine-tuned Ald density on their surface, and nontargeted PEG-PLGA (PP) NPs, are shown in Fig. 1 *A* and *B* and Fig. S1. The physicochemical characteristics and bortezomib drug load of the NPs (Fig. 1 *C* and *D*) were optimized by analyzing a library of NPs formulated (Fig. S2) with varying parameters such as the following: formulation technique, polymer molecular weight, polymer concentration, ratio of organic to aqueous phase, formulation condition, and initial drug feed (Fig. 1*D* and Fig. S2 *C*–*E*). The lead candidate NPs synthesized by single-emulsion method of formulation had optimal sizes in the range of 150–200 nm and nearly neutral to slightly negative ζ potentials (Fig. 1 *C* and *D*, and Fig. S2*C*), and were further standardized to enhance their drug load. To obtain optimal binding to the bone mineral along with maximum stealth properties, we blended varying ratios of the polymers: PLGA-*b*-PEG-Ald (Fig. S1) and PLGA-*b*-PEG for NP formulation (Fig. 1 *A*, *B*, *E*, and *F*). Different ratios of blended polymers altered the Ald content of NPs. We analyzed the stability and size of these NPs in the presence of ions and serum conditions, and the results demonstrated time-dependent increase in NP size, when the content of PLGA-*b*-PEG-Ald polymer in the NPs was higher than 20% (Fig. 1*F*). Thus, it is important to optimize the Ald content of NPs for effective bone binding with maintenance of stealth properties, which ensures enhanced bone homing of NPs, in vivo.

Encapsulation and Release of Bortezomib from NPs. The ability of the NPs to encapsulate high loads of drug and subsequently release the drug in a controlled manner was significantly affected by PLGA molecular weight and content in the NPs, in addition to the formulation techniques and conditions, as investigated by using HPLC. In the case of NPs formulated by the solvent dispersion method, the hydrodynamic diameter (dynamic light

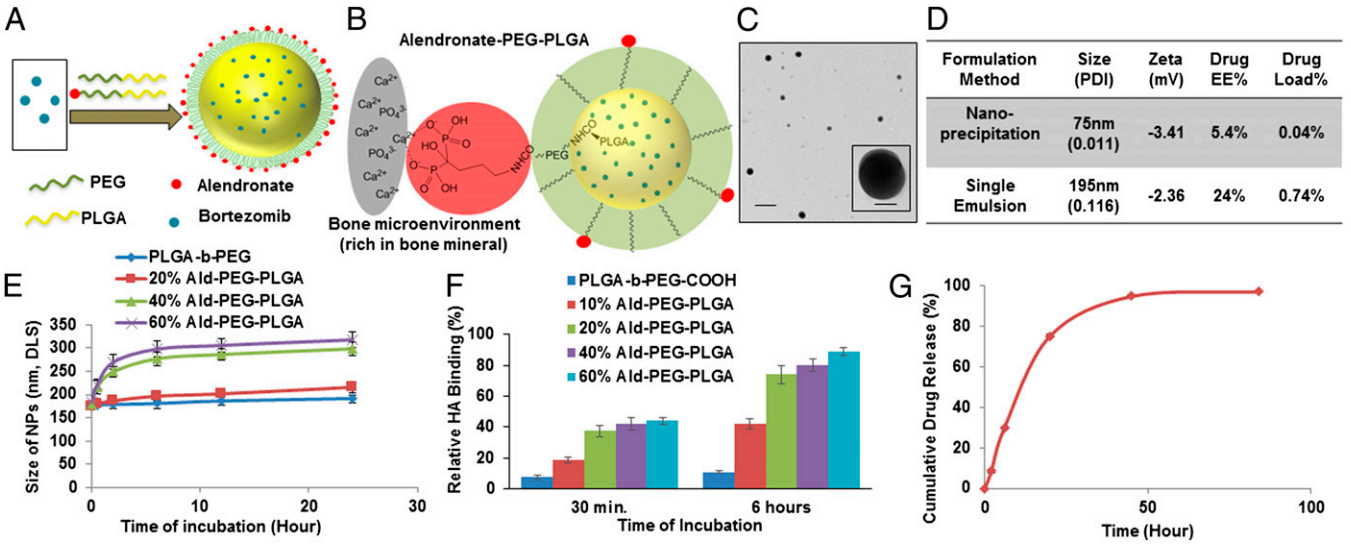

Fig. 1. Design, engineering, and characterization of NPs for bone targeting. (*A*) Schematic illustration of alendronate-conjugated PEG-PLGA (Ald-PP) NPs synthesized by blending polymers (PLGA-*b*-PEG-Ald and PLGA-*b*-PEG) in varying ratios and encapsulating the drug bortezomib. (*B*) Schematic representation of the mechanism of affinity of Ald-PP NPs with bone mineral (gray, bone mineral; red, Ald; green, PEG; yellow, PLGA). (*C*) Representative TEM image of Ald-PP NPs (single emulsion), negatively stained, imaged at 80.0 kV. (Scale bars: 500 nm; *Inset*, 100 nm.) (*D*) Physiochemical characteristics of Ald-PP NPs. (*E*) Size of the Ald-PP NPs (single emulsion) with varying content of polymer PLGA-*b*-PEG-Ald, in presence of serum, with time. (*F*) Quantitative evaluation of HA binding of NPs (single emulsion) with varying content of PLGA-*b*-PEG-Ald polymer. PLGA-*b*-PEG (-COOH terminated) polymeric NPs were used as control. (*G*) Release kinetics of encapsulated drug bortezomib from the Ald-PP NPs (single emulsion), in physiological ionic and temperature conditions.

scattering) was small (80–100 nm), with lower encapsulation efficiency (5–8%) along with lower drug loading (0.04–0.09%). In the case of the single-emulsion NPs with optimal polymer weight (M_r, 45 kDa) and formulation conditions, the NP drug load was enhanced 16- to 20-fold and the release kinetics showed sustained drug release (Fig. 1 *D* and *G*, and Fig. S2 *B*, *D*, and *E*). This can be attributed to the dispersion of the encapsulated drug from the PLGA core of NP by diffusion and polymer degradation. The NPs protect the drug from the external environment, and increase its blood circulation time, thereby increasing the drug content at the target site.

In Vitro Bone Targeting of NPs. Bone microenvironment is rich in HA, particularly the sites of metastatic lesions, where the bone turnover is high, and to investigate the bone affinity of the Ald-PP NPs (single emulsion), we performed the HA binding assay (Fig. 1*F*), in comparison with nontargeted PP NPs (Fig. 2*A*). The Ald-PP NP solution on incubation with HA in any form (NPs, microparticles, or bone chips) showed immediate binding (Figs. 1*F* and 2 *B*–*D*). The results demonstrated a significant rise in the HA binding of NPs as the content of PLGA-*b*-PEG-Ald polymer in the NPs was increased from 0% to 20%. This trend plateaued on further increase of PLGA-*b*-PEG-Ald polymer content in NPs to 40% or 60% (Fig. 1*F*). Thus, optimized, targeted Ald-PP NPs with effective HA binding had 20% PLGA-*b*-PEG-Ald polymer, for all studies thereafter. The HA affinity of targeted NPs was also confirmed by the following: transmission electron microscopy (TEM) using HA in NP form (Fig. 2*B*); scanning electron microscopy (SEM) using HA generated from simulated body fluid (Fig. 2*C*); and fluorescence microscopy using bone fragments (mice skull bone, ex vivo) with fluorescently labeled NPs (Fig. 2*D*). Nontargeted PP NPs did not show any specificity or binding to HA, in any form (Fig. 2 *B*–*D*). These results confirmed the impact of the design of our engineered targeted NPs in bone mineral binding and the differential binding of targeted NPs facilitated the next in vivo experiments.

In Vivo Biodistribution Studies of NPs. The bone-homing ability of targeted Ald-PP NPs (single emulsion) was investigated by conducting biodistribution studies using fluorescently labeled NPs (PLGA-Alexa$_{647}$) tracked with an in vivo imaging system (IVIS) in mice (Fig. 2 *E* and *F*), after i.p. injection. At 24 h, mice injected with targeted NPs showed increased retention in areas of the spleen, femur, spin, skull, and lymph nodes (Fig. 2*E*). The higher retention of targeted NPs compared with nontargeted NPs was confirmed by quantifying the total radiance efficiency of the mice body image (Fig. 2 *G* and *H*). For further examination of the retention and bone-homing ability of targeted NPs, the dissected femur and spine of the mice were sectioned, counterstained with DAPI, and imaged with a fluorescence microscope. Quantification of the NP in bone sections, as measured by the fluorescence (Alexa$_{647}$), revealed 9.6-fold increased accumulation of targeted NPs compared with nontargeted NPs (Fig. 2 *F* and *H*, and Fig. S3). Thus, in vivo biodistribution and histology studies complement the in vitro and ex vivo bone-binding results, which highlight the potential of our engineered NPs for targeting bone.

In Vitro Uptake and Efficacy Studies of NPs in Myeloma Cells. Cellular uptake and accumulation of fluorescently labeled Ald-PP and PP NPs (single emulsion) were quantified using flow cytometry, and demonstrated uptake by peripheral blood mononuclear cells (PBMCs), and to a greater extent by myeloma cells (MM1S) (Fig. 3*A*) with no observable cytotoxic effects. These NPs were also visualized in MM1S cells as demonstrated by using fluorescence microscopy after 24 h of in vitro culture with NPs (Fig. 3*B*). Bortezomib-loaded PP NPs (Bort-NPs) were then assessed for their ability to induce apoptosis and inhibit MM growth in vitro. Apoptosis analysis assessed by Annexin-V/propidium iodide (PI)

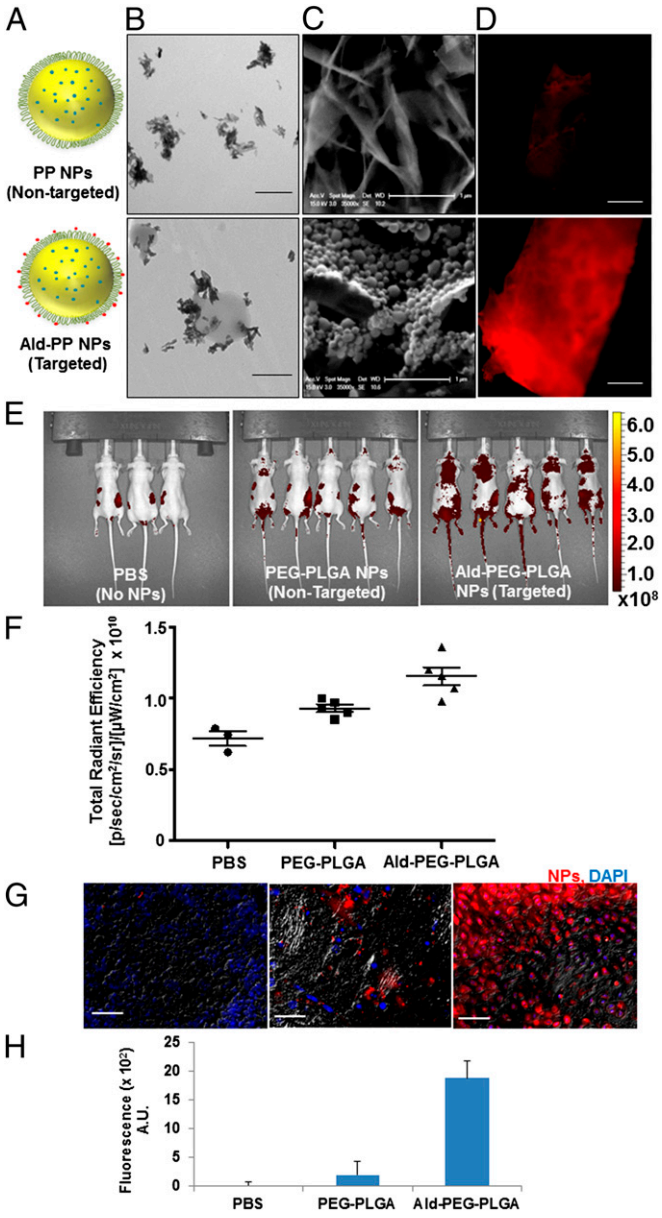

Fig. 2. Bone-targeting ability of Ald-PP NPs (single emulsion). (*A*) Schematic illustration of bone-targeted Ald-PP NPs and nontargeted PP NPs. (*B*) Representative TEM image of Ald-PP (*Lower*) NPs surface interactions with HA rods, which is not observed in case of nontargeted PP NPs (*Upper*). (Scale bar: 500 nm.) (*C*) Representative SEM image of interaction of NPs (targeted Ald-PP: *Lower*; nontargeted PP: *Upper*) with crystalized HA (scale bar: 1 μm) after incubation with NP solution and washing. (*D*) Representative fluorescence image of bone fragment after incubation with fluorescently labeled NP solution (targeted Ald-PP: *Lower*; nontargeted PP: *Upper*) (ex vivo), and washing. (Scale bar: 500 μm.) (*E*) Whole-body mice imaging (IVIS), where targeted NP (*Right*) clearance is compared with nontargeted NPs (*Center*) and PBS (*Left*) (24-h time point, i.p. injection). Scale represents luminescence signal from Alexa$_{647}$-labeled NPs, representing NP biodistribution. (*F*) Total fluorescence quantified in the region of interest of IVIS images from *E*. (*G*) Representative images of bone histology in merged channels (405: DAPI; 647: NPs; and bright field) for PBS (*Left*), nontargeted NPs (*Center*), and targeted NPs (*Right*). (Scale bar: 100 μm.) (*H*) Quantification of NP homing as measured from the bone (femur and spine) histology by fluorescence intensity (average) quantification in the 647 channel in multiple sections of bone, covering entire region representatively, in different mice (n = 3).

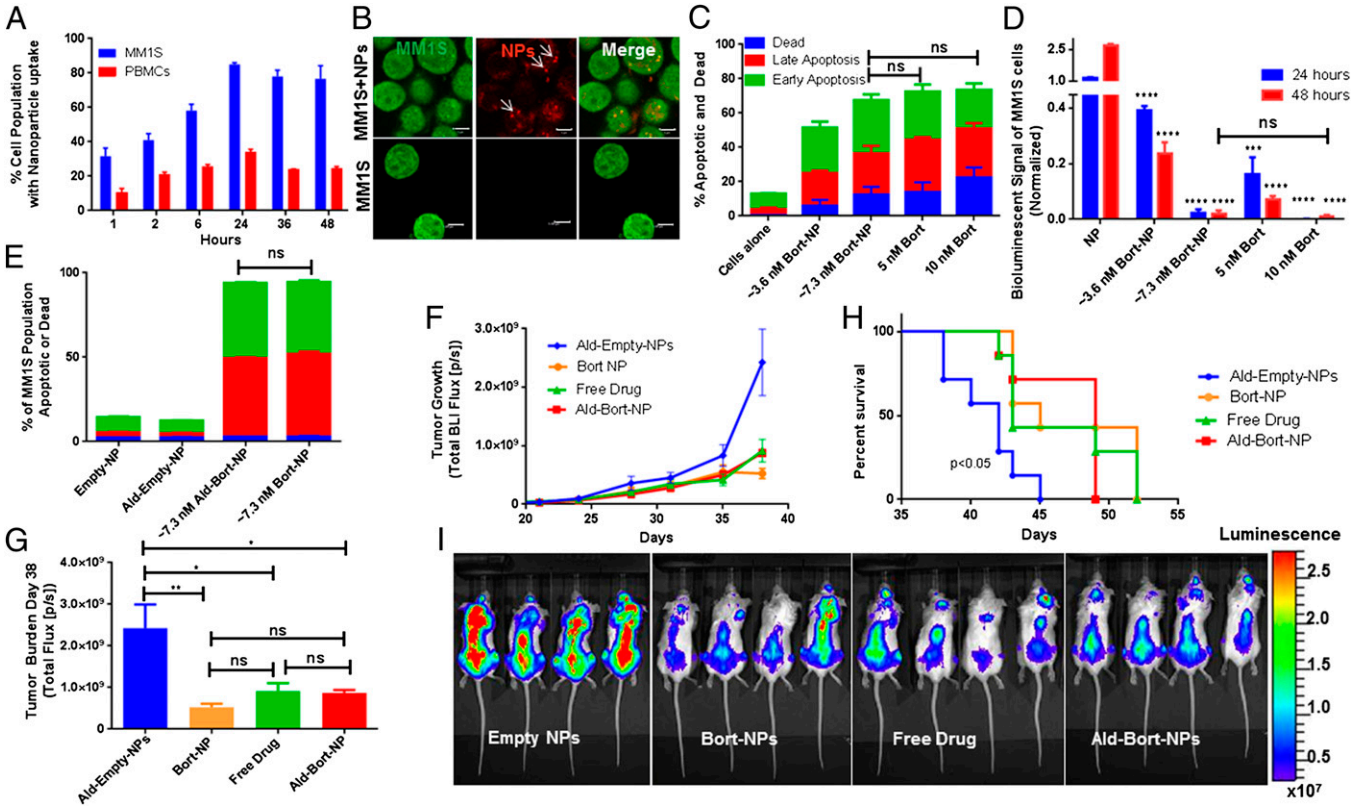

Fig. 3. In vitro and in vivo efficacy of NPs (single emulsion). (*A*) Cellular uptake of NPs during coculture with myeloma (MM1S) cells and peripheral blood mononuclear cells (PBMCs). (*B*) $Alexa_{647}$-labeled NPs imaged in GFP^+ MM1S cells using fluorescence confocal imaging. Bort-NPs induced apoptosis and death in MM1S cells (24 h) (scale bar: 5 μm) (*C*) as measured by Annexin-V/PI flow cytometry; and (*D*) bioluminescent signal quantification of GFP^+Luc^+ MM1S cells (24, 48 h). In *C* and *D*, cells were treated with effective bortezomib concentrations of ∼3.6 or ∼7.3 nM (Bort-NPs) or free drug (5 or 10 nM). *T* tests evaluating efficacy of treatments vs. NP controls at same time point show equivalent efficacy of 7.3 nM Bort-NPs and 10 nM Free Drug. (*E*) Annexin-V/PI flow cytometry of GFP^+ MM1S cells treated with Empty-NPs, Ald-Empty-NPs, ∼3.6 nM Ald-Bort-NPs, and ∼7.3 nM Bort-NPs after 24 h. The stacked bars represent means ± SEM. (*F–I*) Mice injected with GFP^+Luc^+ MM1S cells, treated with Ald-Empty-NPs, Bort-NPs, Free Drug, and Ald-Bort-NPs twice a week, starting at day 21 after tumor cell injection ($n = 7$). (*F*) BLI flux measuring tumor burden in mice from day 21 to 38. (*G*) Quantification of BLI at day 38. (*H*) Survival data for mice treated with Bort-NPs, Ald-Bort-NPs, Free Drug, or NP controls. (*I*) Representative BLI images of mice at day 38 from the four groups. Scale represents luminescence signal from Luc^+ MM1S cells, quantifying tumor burden.

staining and flow cytometry showed similar induction of apoptosis of MM1S cells at 24 h using bortezomib-loaded NPs or free drug (Fig. 3*C*). Bioluminescent quantification of cell numbers also demonstrated similar in vitro bortezomib efficacies when delivered in NPs or as a free drug, with no significant difference found between 7.3 nM Bort-NPs and 10 nM free drug at 48 h. All treatments significantly decreased MM1S cell numbers at all time points. These results illustrate the ability for NPs to effectively deliver bortezomib to inhibit myeloma growth in vitro (Fig. 3*D*). The addition of Ald did not change the efficacy of Bort-NPs in inducing apoptosis, and both drug-free PP and Ald-PP NPs were nontoxic, as expected (Fig. 3*E*) (10, 11).

NPs Inhibit MM Growth in Vivo. In the next set of experiments, we used a MM1S xenograft osteolytic bone disease model (22) where GFP^+Luc^+ MM1S cells were injected into the tail vein of SCID-beige mice, treated with NPs and controls, and measured for tumor burden using bioluminescent imaging (BLI) and survival. MM1S tumor burden was significantly decreased by Ald-Bort-NPs, Bort-NPs (Ald free), and Free Drug compared with Ald-Empty-NPs (no-drug Ald-PP NPs) at day 38 (Fig. 3 *F* and *G*). These data indicate that Ald-Bort-NPs and Bort-NPs were able to reduce tumor burden to the same extent as Free Drug. There was also a significant increase in the survival for mice treated with Ald-Bort-NPs, Bort-NPs, and Free Drug, compared with Ald-Empty-NPs (Fig. 3 *H* and *I*). This evidence demonstrates that bortezomib delivery with NPs works as well as conventional, free drug delivery, in the mice model.

In the treatment study of established myeloma, we believe cancer inhibition was not observed with the use of NPs because, in mice, much of the disease develops outside of the bone marrow niche (circulating and lodged in extramedullary/nonbone locations), which is one of the major differences between mouse myeloma models and the clinical presentation, making inhibition by bortezomib equally efficacious when delivered by any of the compared methods. Conversely, in patients, MM growth is more bone-restricted and treatment with bone-targeting NPs could potentially show increased efficacy vs. free drug or non–bone-targeted NPs by increasing the therapeutic window specifically in the location of the highest MM cell concentration. Furthermore, although we are unable to model peripheral neuropathy in mice due to inherent neurological differences in mice and humans, bone-targeted NPs may potentially improve patient outcomes by decreasing neuropathy from off-target effects of bortezomib.

Bortezomib Increases Osteogenic Differentiation in Vitro and in Vivo. After validating the ability for bortezomib to increase osteogenic differentiation of bone marrow-derived mesenchymal stromal cells (MSCs) in vitro (Fig. S4), we assessed the effects of bortezomib in vivo. Mice were pretreated with Ald-Empty-NPs, Free Drug, or Ald-Bort-NPs for 3 wk, thrice a week, and euthanized thereafter. Bones were analyzed with micro-computed tomography (micro-CT) analysis of femur and tibia, and static bone histomorphometry of the tibia. We observed significantly increased bone trabecular volume, as demonstrated in Von Kossa-stained tibia slides (Fig. 4*A*),

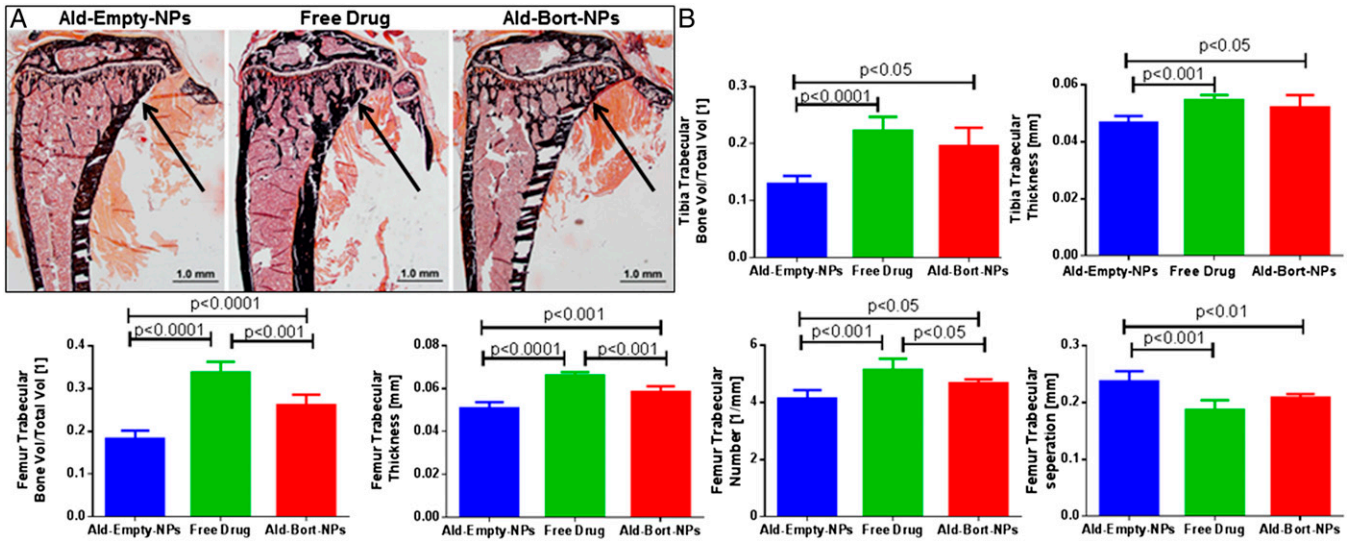

Fig. 4. In vivo effects of bortezomib NPs (single emulsion) on bones. Mice were pretreated for 3 wk, with Ald-Empty-NPs, Free Drug, or Ald-Bort-NPs. Static bone histomorphometry and micro-CT done on these samples show an increase in bone formation markers for the bortezomib-treated groups. (*A*) Representative images from static histomorphometry from each group shown by Von Kossa staining. Trabecular bone volume was increased in Free Drug and Ald-Bort-NP groups compared with that of Ald-Empty-NP group, as indicated by arrows. (*B*) Micro-CT analysis demonstrated significantly higher bone in Ald-Bort-NPs and Free Drug compared with that of Ald-Empty-NPs in terms of the following: tibia trabecular bone volume per total volume, tibia trabecular thickness, femur trabecular bone volume per total volume, femur trabecular thickness, femur trabecular number, and femur trabecular separation.

and quantified with micro-CT in the tibia and femur and histomorphometry of the proximal tibia (Fig. 4*B*). Histomorphometric analysis also demonstrated significant increases in osteoid thickness and decreases in several bone resorption parameters in mice treated with either Ald-Bort-NPs or Free Bortezomib, vs. Ald-Empty-NPs (Table S1), with both changes (increased osteoblastic activity and decreased osteoclastic activity) contributing to the increased bone volume. Significantly higher bone metrics were also observed in Ald-Bort-NPs and Free Drug compared with Ald-Empty NPs using micro-CT quantification, in terms of tibia trabecular bone volume per total volume, tibia trabecular thickness, and femur trabecular bone volume per total volume, femur trabecular thickness, femur trabecular number, and femur trabecular separation (Fig. 4*B*). This evidence demonstrates the ability for both free bortezomib and bortezomib-loaded NPs to increase volume of bone, and number of trabeculae, in vivo over a 3-wk pretreatment period. We next investigated the consequences of these treatments on the growth of MM.

Pretreatment with Bone-Targeted, Bortezomib NPs Inhibits Myeloma Growth. To examine whether modulating the bone marrow niche before metastasis occurs can prevent/delay disease progression, mice were pretreated with Ald-Bort-NPs, Ald-Empty-NPs, or Free Drug for 3 wk, thrice a week. This allowed for the modulation of the bone microenvironment before the arrival of cancer cells. They were then injected with GFP^+Luc^+ MM1S cells into the tail vein and assessed for tumor progression. Of great importance was our observation that pretreatment with Ald-Bort-NP significantly inhibited myeloma growth as observed with significantly lower BLI signal compared with the Free Drug and Ald-Empty-NP groups ($P < 0.05$) (Fig. 5 *A* and *C*). Survival time was also significantly increased in the Ald-Bort-NP group with median survival of 41 d,

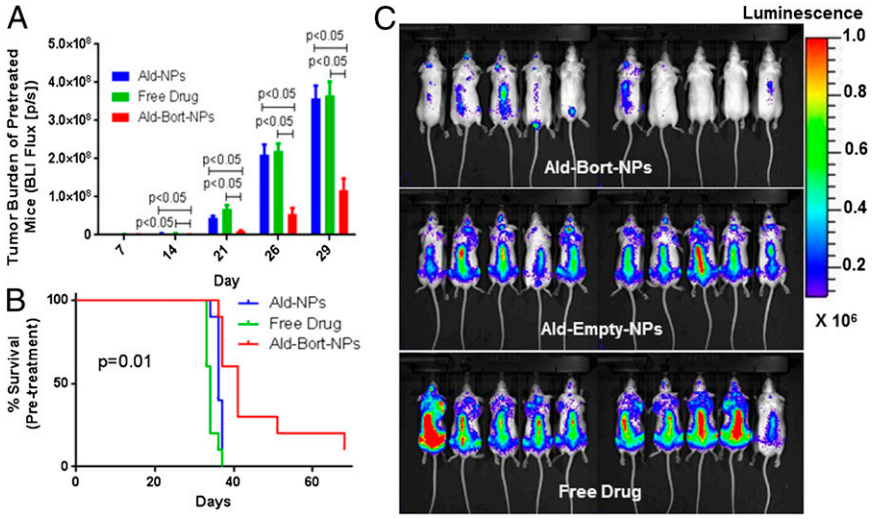

Fig. 5. Pretreatment with Ald-Bort-NPs inhibits myeloma growth better than free drug. (*A*–*C*) Mice were pretreated for 3 wk with Ald-Empty-NPs, Free Drug, or Ald-Bort-NPs and then injected with GFP^+ Luc^+ MM1S cells. (*A*) BLI flux from mice was significantly lower in Ald-Bort-NPs compared with that of Ald-Empty-NPs or Free Drug groups at every day of imaging. (*B*) Survival was also significantly increased in the Ald-Bort-NP–pretreated mice ($P = 0.01$). (*C*) Day 29 images of BLI signal from mice illustrates the reduction of tumor burden in mice pretreated with Ald-Bort-NPs ($n = 10$).

compared with just 34 or 36 d in the Free Drug group, and Ald-Empty-NP groups, respectively (Fig. 5*B*). In a second in vivo study (Fig. S5), we confirmed that pretreatment with bone-homing bortezomib NPs improved survival compared with pretreatment with nontargeted bortezomib NPs. Both treatments significantly improved survival compared with empty-NPs, further confirming that bortezomib NP drug delivery creates a less hospitable bone microenvironment for cancer cells. These results suggests that Ald-Bort-NPs may have the ability to alter the microenvironment to prevent myeloma growth via mechanisms other than increasing in bone volume, trabecular number, or osteoid thickness, and should be explored for their ability to inhibit other bone-metastatic cancers.

Conclusion

In summary, we developed, biodegradable polymeric NPs capable of targeting bone and delivering the payload in a spatiotemporally controlled manner. These NPs were shown to enhance bone homing due to long circulation and bone mineral-targeting capabilities. The bone-targeted NPs with sustained release polymer technology delivered bortezomib to bone marrow microenvironment specifically, to produce the antimyeloma effects similar to a free drug. However, the major drawback of using a free drug, bortezomib, is peripheral neuropathy (5), and the use of our NPs would be hugely beneficial by enabling bone-specific drug delivery, which should drastically decrease these side effects in patients. It is also well known that MM resistance is due to cell dormancy within the bone marrow, and the clonal nature of MM, which is driven by a wide range of interactions, constantly evolving mutations, and heterogeneous abnormalities. However, targeting the microenvironment, on the other hand, translates well to all patients, regardless of the driver mutation. Thus, our NPs, which are specifically designed to home to the bone marrow, release the drug to target both the cancer and the microenvironmental cells. Furthermore, the design of our engineered NP has far-reaching advantages of flexibility of NP design, scalability, biocompatibility and biodegradability, long circulation, sustained drug release, bone-homing property, and fine-tuned components for clinical translation. In the future, this platform could be used in many other cancer models to deliver many different anticancer agents. The results of the present work demonstrate the tremendous potential of the bone-targeted Ald-PP NPs in the pretreatment strategy for modifying the bone microenvironment with suitable drugs to prevent cancer progression and lesion formation, providing a promising nanomedicine approach for MM therapy.

Materials and Methods

(See *SI Materials and Methods* for details.) To optimize NP formulation with suitable physicochemical characteristics, with varying ratios of target ligand (Ald) to PEG density on NP surface, and to maximize the drug load, we prepared a library of NPs, using different polymer molecular weights, blending different ratios of synthesized polymers (Figs. S1 and S2) (23), using different formulation techniques, and varying the conditions of formulations. The affinity of Ald-conjugated NPs (Ald-PP) toward bone mineral (HA) was investigated in comparison with nontargeted (PP) NPs. We studied the in vivo biodistribution of $Alexa_{647}$-labeled Ald-PP NPs with whole-mouse imaging. NPs were injected i.p. and after imaging (1, 24 h), the mouse bones were dissected, sectioned, and imaged for investigation of bone homing of labeled NPs (Fig. S3). We investigated the in vitro efficacy of Bort-NPs by measuring apoptosis via flow cytometry, and bioluminescence assay, where empty NPs, and free bortezomib were the controls (24). The in vivo efficacy studies used female Nod/SCID beige mice in treatment or pretreatment regimes. For treatment studies, mice injected with Luc^+/GFP^+ MM1S cells were randomly divided into four groups ($n = 7$). After injecting cancer cells, on day 21 mice were injected (i.p.) twice a week with 0.5 mg/kg bortezomib (or with an equivalent amount of Ald-Empty-NPs): Ald-Empty-NPs, Free Drug (bortezomib), Ald-Bort-NPs, and nontargeted Bort-NPs, and were imaged twice a week. In the case of NP pretreatment regime, female Nod/SCID beige mice were randomized into three groups ($n = 10$) and injected (i.p.) thrice a week for 3 wk, with 0.3 mg/kg bortezomib or with an equivalent amount of Ald-Empty-NPs. The pretreatment groups were as follows: Ald-Bort-NPs, Free Drug, and Ald-Empty-NPs in study 1 and Ald-Bort-NPs, Ald-Empty-NPs, and Nontargeted Bort-NPs in study 2. After 3 wk, the mice were injected with Luc^+GFP^+ MM1S cell. BLI was performed weekly on these mice and survival was assessed. Additionally, an ex vivo micro-CT analysis and static histomorphometry (25) of mouse bones (femur, tibia, and fibula) were performed after a 3-wk pretreatment period to validate bortezomib-induced increase in osteogenesis. See *SI Materials and Methods, Statistical Analysis* for the details of the statistical analysis.

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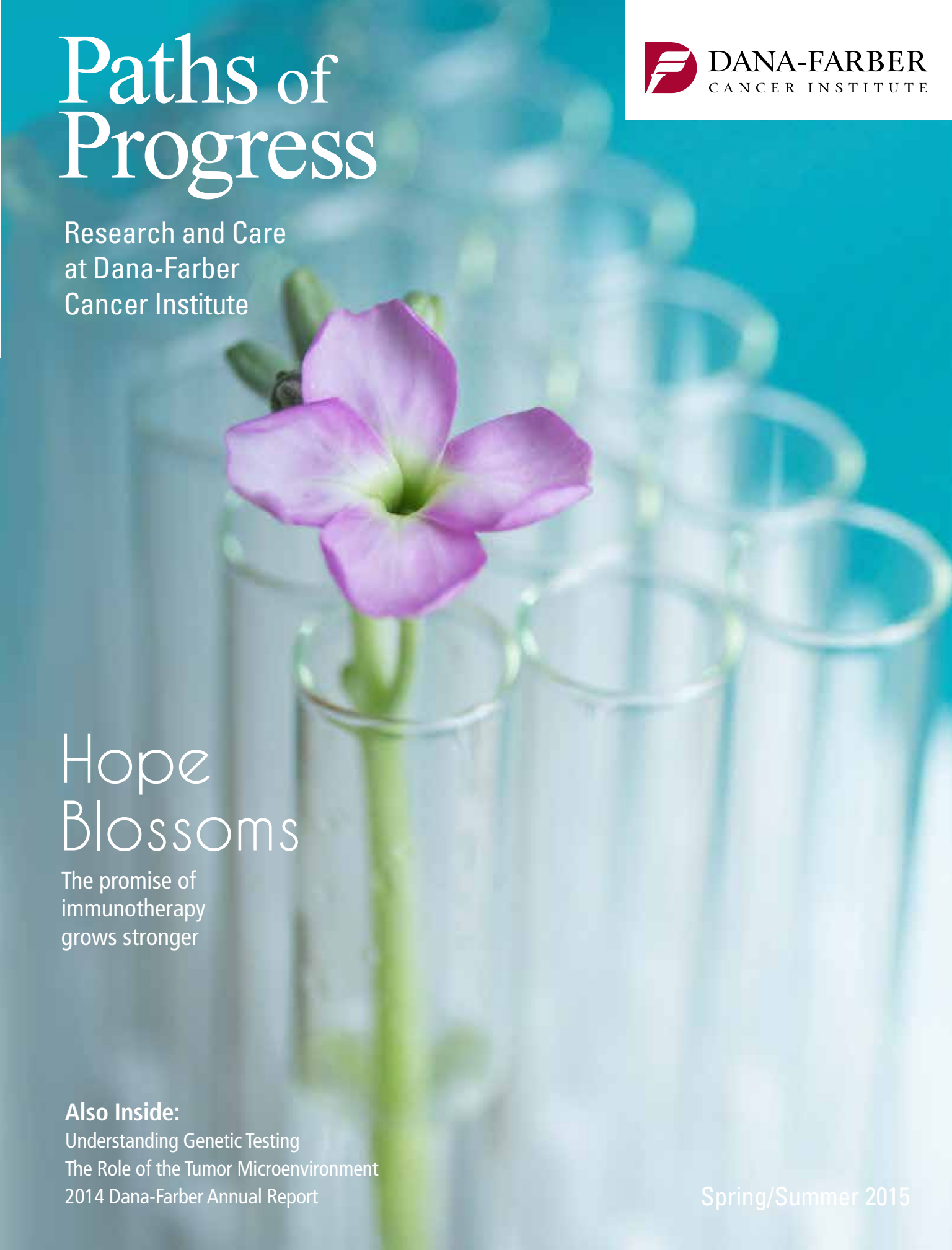
Paths of Progress

Research and Care at Dana-Farber Cancer Institute

DANA-FARBER CANCER INSTITUTE



Hope Blossoms

The promise of immunotherapy grows stronger

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2014 Dana-Farber Annual Report

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Dear Readers,

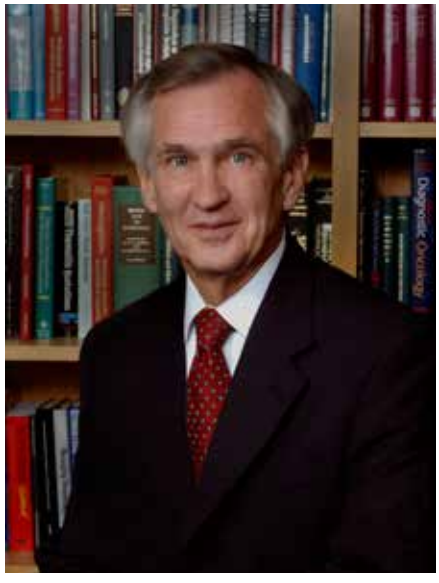
Science, famously, has an impulse to purify, to reduce, to refine. Complex processes are understood by breaking them down into basic components, then allowing those components to interact under carefully controlled conditions – an experiment.

In cancer research, that has traditionally meant growing colonies, or lines, of cancer cells in laboratory glassware. This approach has yielded countless insights into how tumor cells behave and how they might respond to potential therapies, but it doesn't begin to replicate the torrent of biological activity that affects tumors in the body.

For all that cancer seems to resemble a recluse, unmoved by the life around it, tumors are in fact intimately involved with their surroundings. They subvert nearby cells, turning them into accomplices of tumor growth. They hoodwink the immune system into withholding an attack on tumor cells. They initiate changes that make distant organs hospitable sites for metastasis.

This issue of *Paths of Progress* contains several articles on what Dana-Farber scientists are learning about the interactions between cancer and its human environment, and how they're using that knowledge to impede cancer's growth and spread. There's an article on research that seeks to prevent the tumor "microenvironment" – the normal tissue in a tumor's neighborhood – from abetting cancer. Another article describes efforts to expose tumors to an attack from immune system cells in their vicinity. The Science Illustrated section depicts a laboratory model of the bone tissue where multiple myeloma forms.

As cancer research advances, the traditional, reductive style of experimentation is being complemented by a more holistic approach. The results promise to be transformative.

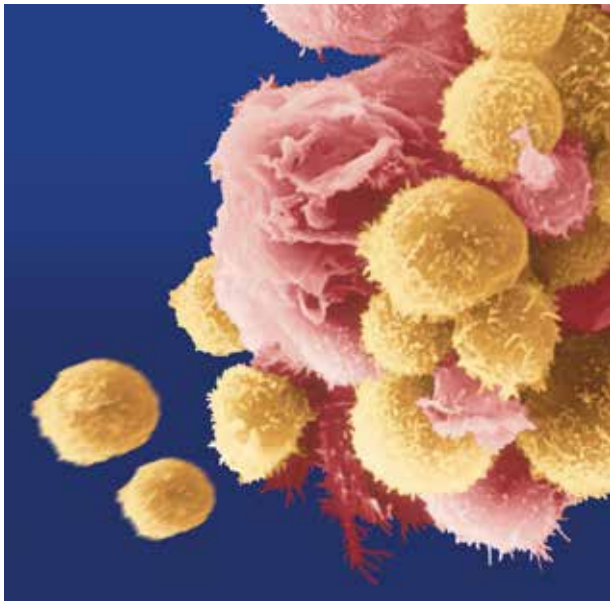


"As cancer research advances, the traditional, reductive style of experimentation is being complemented by a more holistic approach."

– Edward J. Benz Jr., MD

Edward J. Benz Jr., MD
President and CEO, Dana-Farber Cancer Institute

Breakthrough Result for Immunotherapy in Hodgkin Lymphoma



Study results provide strong evidence of the potential of immunotherapies.

A therapy that liberates the immune system to attack cancer cells drove Hodgkin lymphoma into complete or partial remission in 87 percent of patients with resistant forms of the disease who participated in an early-phase clinical trial, investigators at Dana-Farber and partnering institutions report in a study published recently in the *New England Journal of Medicine*.

The results provide some of the most dramatic evidence to date of the potential of therapies that increase the ability of the immune system to kill cancer cells. While clinical trials of such immunotherapies in other cancers have shown them to be highly effective in a subgroup of patients, the new study stands out because nearly all patients benefited from the treatment.

The success of the agent, nivolumab, in this study has prompted the U.S. Food and Drug Administration to designate it a "breakthrough therapy" for treating relapsed Hodgkin lymphoma, and a large, multinational phase 2 trial is now under way.

"What makes these results especially encouraging is that they were achieved in patients who had exhausted other treatment options," said the study's co-senior author, Margaret Shipp, MD, chief, Division of Hematologic Neoplasia at Dana-Farber. "We're also excited by the duration of responses to the drug. The majority of patients who had a response are still doing well more than a year after their treatment."

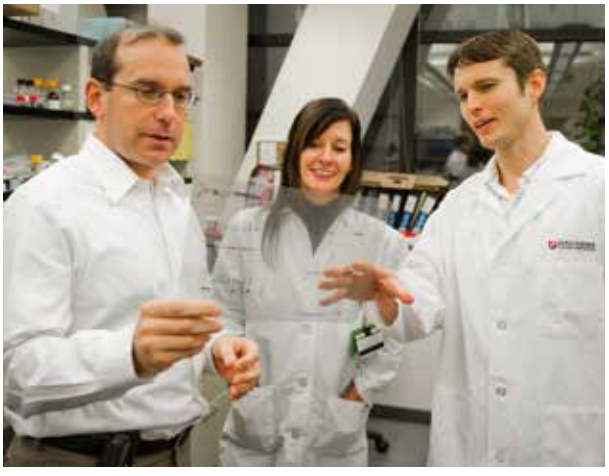
The study involved 23 patients with relapsed or treatment-resistant Hodgkin lymphoma. The patients received biweekly infusions of nivolumab, which is an antibody that blocks a protein called PD-1 on the surface of immune system T cells. Of the 23 patients, 20 had a measurable response to the treatment, with four achieving a complete response – in which no detectable tumor was left – and 16 having a partial response – in which their tumors shrank to less than half their original size. Six months after completing therapy, 86 percent of the patients were alive with continued responses. Most patients continue to do well a year after their treatment.

For the investigators involved in the research, the results, though obtained in a relatively small, phase 1 trial, are compelling. "For someone like myself, in this kind of work, this is the kind of result that you get to see once in your career," said study co-senior author Philippe Armand, MD, PhD, medical oncologist in the Hematologic Oncology Treatment Center at Dana-Farber.

Personalized Test May Help Pinpoint Best Treatments

In a recent study published in the journal *Cell*, Dana-Farber researchers say a novel lab test can predict within less than 24 hours which agent is most likely to work against a particular tumor. The test works by measuring how vigorously tumor cells turn on "self-destruct" signals when exposed to different cancer drugs.

The scientists say this technique could lead to more reliable and rapid tools for "personalizing" cancer treatments. Clinical testing has begun.



Anthony Letai, MD, PhD (left), and his team explore tests that may predict which therapies work best for patients.

A team led by Dana-Farber oncologist Anthony Letai, MD, PhD, reported that the test consistently predicted the "winner" among many drugs tested against a wide variety of cancer cells in the laboratory. In most cases, the answer emerged 16 hours after the anticancer compounds were mixed with tumor cells.

"We demonstrated that [the test] can be exploited to select among many therapies the one that it is best for a single tumor," the researchers wrote. "We also demonstrated that it can select among many patients those most likely to respond to a therapy."

The technique, called Dynamic BH3 Profiling, or DBP, is designed to detect the earliest signs that a cancer cell treated with a drug is beginning to destroy itself through apoptosis, a natural quality-control process that rids the body of unneeded or dangerously abnormal cells.

"This new technique represents a completely novel approach to precision medicine because we can test possible treatment directly on patient samples to guide cancer therapy," said Joan Montero, PhD, the first author on the report and a researcher in the Letai group.

Precision Cancer Medicine at DF/BWCC

Dana-Farber/Brigham and Women's Cancer Center (DF/BWCC) now offers a Precision Cancer Medicine website at *www.precisioncancermedicine.org* created to help patients and physicians learn more about this exciting new field. The site describes an evolving approach to cancer care that seeks to leverage rapidly expanding knowledge about the molecular basis of cancer to more precisely direct therapy.

Clinicians at DF/BWCC are now armed with specialized tests, creating a precise tumor profile of genetic changes for each patient's cancer that makes it possible to identify the most important mutations. The results of these tests can aid us in selecting specific therapeutic agents to precisely treat each individual's cancer.

Learn more at *www.precisioncancermedicine.org*.

Social Media

Visit our Vine page to find quick tips on healthy living and highlights from Dana-Farber events: *http://vine.co/DanaFarber*

Connecting with Dana-Farber has never been easier. Find links to all of our social media sites: *www.dana-farber.org/socialmedia*

Study Finds Promising Drug Target for Some Cancers

Rafael Ceccaldi, PhD (left), and Alan D'Andrea, MD, led a study that revealed an enzyme called POLQ is a promising drug target in some breast and ovarian cancers.

A Dana-Farber study published in the journal *Nature* helped uncover a promising drug target in some breast and ovarian cancers. Research by Dana-Farber scientists indicates that the pool of patients who can benefit from the drug olaparib is potentially much wider than previously known – and the new study offers a means of identifying them. Olaparib was recently approved by the Food and Drug Administration's for treating ovarian cancer patients with inherited mutations in *BRCA1* or *BRCA2* genes.

The study found that an enzyme called polymerase θ (or POLQ) is the active agent in the protein "pathway" that olaparib targets within tumor cells. The finding suggests that breast and ovarian cancer patients whose tumor cells carry abnormally high levels of POLQ are likely to respond to the drug – and POLQ itself is an inviting target for future therapies.

"Although olaparib is often effective in [women who have inherited mutations in *BRCA1* or *BRCA2*], we haven't known precisely how it works," says Alan D'Andrea, MD, co-director of Dana Farber's Gene Therapy Center and senior author of the study. "By uncovering part of the biological machinery that olaparib operates on, we now have a rationale for making it and similar drugs available to a broader population of patients."

Olaparib belongs to a class of drugs known as PARP inhibitors, which target one of the mechanisms cells use to repair certain kinds of damage to DNA. While researchers knew that olaparib targets the PARP pathway, they didn't know precisely how it and other PARP inhibitors work. To find out, first author Raphael Ceccaldi, PhD, of D'Andrea's lab, probed cancer cells for proteins whose levels shot up when *BRCA1* or *2* was mutated. The protein that underwent the biggest increase was POLQ.

"The discovery that cancer cells with *BRCA* mutations are utterly dependent on POLQ for their continued survival – and that normal cells are not – suggests that POLQ could be an ideal target for novel therapies," D'Andrea say. "We're working on developing such therapies now."

IN THE NEWS

Boston magazine named **57 physicians and surgeons affiliated with Dana-Farber** to its annual Top Doctors guide. Drawing from a Castle Connolly Medical Ltd. database, the list consists of 649 Boston-area physicians from more than 50 medical specialties. It is online at *bostonmagazine.com*.

David Williams, MD, a leader of Dana-Farber/Boston Children's Cancer and Blood Disorders Center, was recently inducted as president of the American Society of Hematology (ASH), the world's largest professional society of hematologists. He's joined in his leadership role at ASH by Dana-Farber's **Kenneth C. Anderson, MD,** director of Dana-Farber's Jerome Lipper Multiple Myeloma Center and LeBow Institute for Myeloma Therapeutics, who was elected vice president of ASH.

David G. Nathan, MD, president emeritus of Dana-Farber and physician-in-chief emeritus of Boston Children's Hospital, received the first Lifetime Impact Award from Boston Children's. The award recognizes a career spent accelerating innovation in pediatric medicine.

Many Women Don't Know the Basics of Their Breast Cancer

A recent Dana-Farber study reported in the journal *Cancer* found a striking lack of knowledge among breast cancer patients about the basic characteristics of their disease – how advanced it is (stage), whether it is fueled by estrogen, whether it can be treated with trastuzumab (also called Herceptin), and the grade assigned by pathologists.

All these factors are taken into account when treatments are recommended, and researchers suggest that patients who fully understand their cancer may be more likely to adhere to treatment regimens.

"We were really surprised by the results," says Rachel Freedman, MD, MPH, a medical oncologist in the Susan F. Smith Center for Women's Cancers who is first author of the report.

The study is believed to be the first to pose these questions, said Freedman. Although past research has examined cancer patients' general knowledge about basic treatment rationales and reasons for screening, this is the first study to examine how much women know about their own cancers.

Phone interviews were carried out with 500 women in the California Cancer Registry who had undergone surgery for breast cancer. The results found that 56 percent of women were correct on their ER status, 58 percent reported the correct HER2 status, 57 percent were correct about the cancer's stage, and 20 percent reported the correct grade. Only 8 percent were correct on all four questions.

Rachel Freedman, MD, MPH, examines patient knowledge.

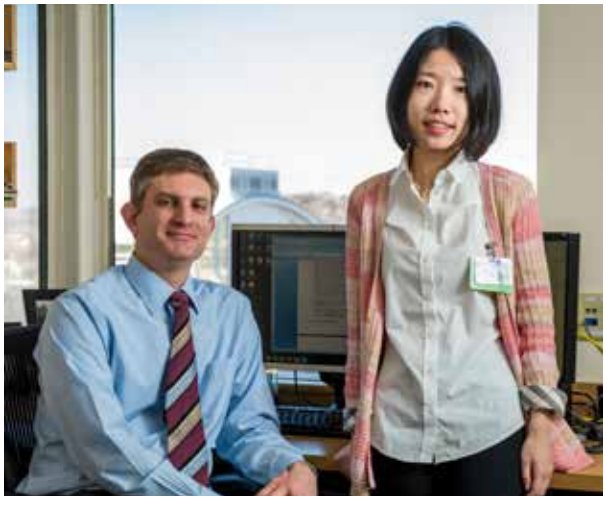
"Of all of these factors, a tumor's grade is likely the least important element for patients to know, although physicians use grade to make decisions about treatments," Freedman says.

Freedman plans to study the effect on patient knowledge of doctors and health care providers' different styles of communicating the facts. She has considered possible interventions, such as patient videos, smartphone apps, and checklists.

Diabetes Affects Pancreatic Cancer Survival

Pancreatic cancer often offers doctors few clues about how patients with the disease are likely to fare, but in a new study Dana-Farber scientists have found one important indicator: a long history of diabetes.

By analyzing data on more than 1,000 patients with pancreatic cancer who participated in long-running health studies, the researchers found that patients with long-term diabetes (more than four years) had a shorter median survival time than those who weren't diabetic. By contrast, patients with recent-onset diabetes (less than four years) survived roughly the same amount of time as nondiabetics did.



Brian Wolpin, MD, and Chen Yuan (right) led a study of how diabetes may impact survival time.

To confirm the findings, researchers analyzed data from nearly 400 pancreatic cancer patients treated at Dana-Farber. Again, long-standing diabetes was associated with shorter survival – nine months, versus 13 months for nondiabetic patients.

"Diabetes can lead to other health problems, such as heart and kidney disease," says Dana-Farber's Brian Wolpin, MD, MPH, who, with Chen Yuan, led the study, published in the *Journal of Clinical Oncology*."However, we found that the difference in survival rates remained even after we adjusted for the presence of these conditions."

The authors theorize that long-term changes in metabolism due to diabetes might affect the genetic makeup of pancreatic tumors, rendering them more aggressive. They are testing this by collecting tumor samples from a large group of patients and analyzing genetic changes. This may identify therapies that are more effective in patients with diabetes.

A closer look at a few of the thousands of words associated with the complex fields of cancer medicine and research.

***BRCA1*:** A gene that repairs DNA damage in cells, which in turn can help prevent tumors. A person who inherits a mutated version of the *BRCA1* gene may have a higher risk for breast, ovarian, and other types of cancer.

Glioblastoma: A fast-growing tumor that arises in the supportive tissues of the brain and spinal cord.

Monoclonal gammopathy of undetermined significance (MGUS): A condition in which a person has a higher-than-normal level of a monoclonal protein, or M protein, in the blood. Patients with MGUS have a higher risk for developing cancer.

Multiple myeloma: A type of cancer that begins in the plasma cells of bone marrow. (Plasma cells are the white blood cells that produce antibodies.)

INTERVIEW BY RICHARD SALTUS

When oncologists who treat blood cancers needed a way to get genetic test results faster, R. Coleman Lindsley, MD, PhD, and his colleagues at Dana-Farber/Brigham and Women's Cancer Center (DF/BWCC) got creative. They devised an all-in-one, in-house test called Rapid Heme Panel (RHP) that can deliver results in less than five business days.

RHP helps doctors identify the genetic mutations driving a patient's leukemia, lymphoma, or other hematological malignancy. The streamlined test debuted at DF/BWCC in 2014.

Why was there a need for an improved testing method?

Before RHP, samples of a patient's cancer were shipped to several different labs and tested for mutations in just a few selected genes. It took a week or two to get those limited results back. In treating aggressive blood cancers, decisions often need to be made within days of diagnosis.

What are RHP's advantages?

RHP can test for a large number of mutated genes at one time and return clinical results quickly. Now, one sample is collected, tested locally, and the results are reported into the patient's record in five business days or less. Currently, RHP tests for mutations in a panel of 95 genes involved in various blood cancers. As new mutated genes are discovered and new clinical data emerge, we continue to update the test.

Which diseases are currently being assessed with RHP?

These include myeloid malignancies (such as acute myeloid leukemia, myelodysplastic syndromes, and myeloproliferative neoplasms), and lymphoid malignancies (such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, hairy cell leukemia, and non-Hodgkin lymphoma).

How has RHP influenced patient care?

RHP is now fully integrated into clinical practice. For example, for patients newly diagnosed with acute leukemia, RHP results give us important information for treatment decisions. When patients have unexplained abnormalities in blood counts, RHP can identify mutations that help make an accurate diagnosis. And when patients are treated for leukemia relapse, the test is critical for identifying the best options for enrollment in clinical trials based on the genetic mutations involved.

What were the challenges in creating this test?

The test uses next-generation sequencing to scan a large panel of genes commonly mutated in blood cancers. All the steps in the process had to be made robust and efficient in order to achieve swift turnaround of accurate results. We relied heavily on the collective expertise of clinicians and researchers in the DF/BWCC community to build the interpretive component of the test.


HOPE

BLOSSOMS

BY ROBERT LEVY

This is a story about the velocity of an idea – a discovery whose potential to improve cancer treatment practically leapt from the test tube.

The groundwork was laid in the 1990s, when scientists learned that human cells carry certain proteins on their surface that enable them to escape attack from the body's immune system. That was followed by the discovery by Dana-Farber scientists that many cancer cells wear one of those same proteins, called PD-L1 – part of an elaborate masquerade that allows the cancer cells to live and multiply without harassment from the immune system.

The implications of that finding, published in 2001, were self-evident: find a way to block PD-L1, or the proteins on immune system cells that "see" PD-L1, and the command that once prevented an immune system attack on cancer would be lifted. Pharmaceutical companies, once skittish about investing in immunotherapies for cancer (agents that sic the immune system on tumor cells), began working on them in earnest.

The first clinical trial of a PD-L1-blocking drug began in 2008 in patients with advanced blood cancers. By the end of 2014, roughly a dozen trials of PD-L1 blockers had been completed and about 50 more were under way at more than two dozen medical centers across the country, involving thousands of patients with a range of different types of cancers.

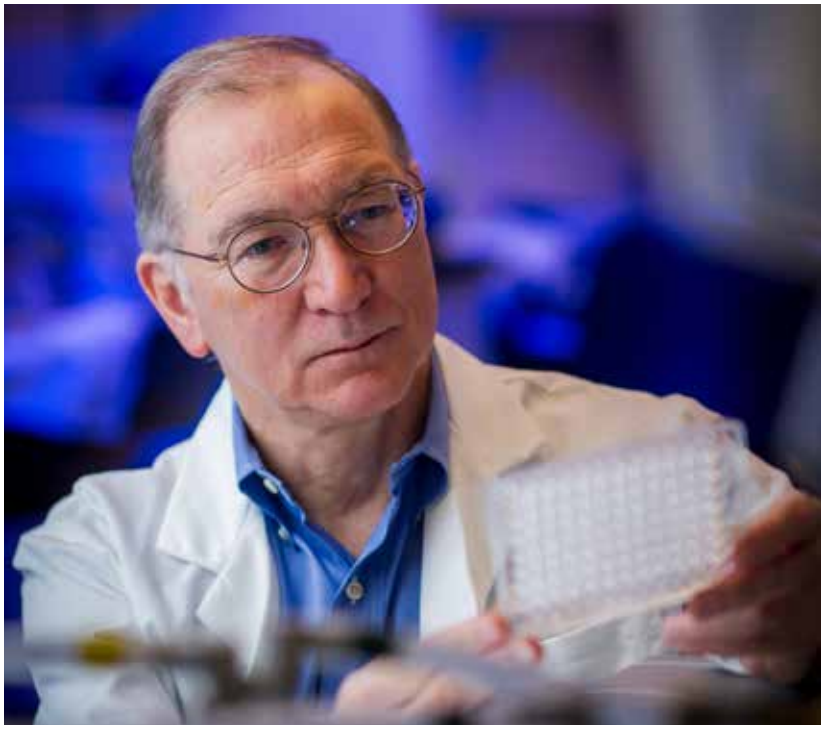
These statistics say much about the promise of this form of immunotherapy. First, they indicate that a substantial number of participants in these trials have benefited from it. (New trials wouldn't be opening at this pace if the treatment wasn't already showing significant signs of success.) Second, they suggest that, unlike some other drug agents, PD-L1 blockers can be effective against multiple types of cancer.

"It makes sense to test these agents in every form of cancer."

– Gordon Freeman, PhD

"It makes sense to test these agents in every form of cancer," says Dana-Farber's Gordon Freeman, PhD, whose lab discovered that PD-L1 resides on normal cells as well as some cancer cells, and that blocking it can provoke an immune system attack on tumors.

This particular type of therapy goes by the name immune checkpoint blockade. "Checkpoint" refers to the encounter between immune system T cells – which patrol the body relentlessly for



Discoveries by Gordon Freeman, PhD, are uncovering cancer's interactions with the immune system, leading to tests of potential new therapies for patients.

signs of infection or other disease – and the PD-L1 protein on tumor cells. T cells use a protein on their own surface, called PD-1, to probe cancer cells for PD-L1 (and a closely related protein, PD-L2). When they find it, they courteously pass by, leaving the tumor cells free to go about their cancerous business. But when a drug agent blocks that signal, the T cells, no longer misled by PD-L1 and PD-L2, rally an immune system attack on the cancer. "This is a really different strategy," says Freeman. "Don't poison the cancer cell but let the immune system directly kill it."

The early rounds of clinical testing of PD-1/PD-L1 checkpoint inhibitors suggest the arrival of a major addition to the anti-cancer arsenal. The inhibitors, which are made from natural human antibodies, work better in some types of cancers than others, but a distinctive pattern has emerged from the trials conducted so far: For patients who do benefit from these agents, the benefits tend to last for years – in some cases, it appears, indefinitely.

One of the most dramatic examples comes from a clinical trial led by F. Stephen Hodi, MD, director of the Melanoma Center at Dana-Farber/Brigham and Women's Cancer Center (DF/BWCC). "Since the year 2000, more than 2,000 patients with metastatic melanoma have been treated with ipilimumab, a drug that blocks an immune checkpoint known as CTLA-4," Freeman relates. "About 20 percent benefited from the drug. The vast majority of them are alive today."

Margaret Shipp, MD (left), and Phillippe Armand, MD, PhD, led a successful early trial of a PD-1 blocker in patients with Hodgkin lymphoma.

The record of clinical research in PD-1/PD-L1 inhibitors is much briefer than that of CTLA-4 inhibitors and is, in many respects, just beginning to be written. But many of the results are of the sort that led *Science* magazine to dub this form of immunotherapy the "Breakthrough of the Year" for 2013.

Hodgkin Lymphoma

In a phase 1 clinical trial led by Dana-Farber's Phillippe Armand, MD, PhD, and Margaret Shipp, MD, investigators tested the PD-1 blocker nivolumab in 23 patients with Hodgkin lymphoma (HL) who had exhausted numerous other treatment options, often including stem cell transplant. Within two to six months, 87 percent of participants experienced a full or partial remission of the disease. The majority of them were still doing well a year after treatment, when the results were published in the *New England Journal of Medicine.*

"HL was a particularly attractive target for PD-1 blockade," Shipp says. "The tumor cells often carry a genetic abnormality that causes them to produce large amounts of PD-L1, and the tumor tissue teems with ineffective immune system cells. Blocking PD-1 is a way to restore their effectiveness." The study findings prompted the U.S. Food and Drug Administration (FDA) to designate nivolumab a "breakthrough therapy" for relapsed HL, and a multinational phase 2 trial is now under way (see page 2).

Glioblastoma

When researchers tested immune checkpoint inhibitors in mice with glioblastoma – an incurable form of brain cancer – the results were hardly ambiguous. Half of the mice that received a PD-1 antibody were long-term survivors: after 50 days, they showed no evidence of tumor in their brain. In mice that received antibodies against PD-L1 and CTLA-4, 25-30 percent were considered cured, says David Reardon, MD, who led the experiments with Prafulla Gokhale, PhD, Sarah Klein, Scott Rodig, MD, PhD, Keith Ligon, MD, PhD, Shakti Ramkissoon, MD, PhD, and Gordon Freeman. These and other results led to the recent opening of three clinical trials (two led by Reardon) of these agents in human patients.

Kidney Cancer

DF/BWCC investigators opened their first clinical trial of a PD-1 and PD-L1 blocker for patients with kidney cancer five years ago. About 20-25 percent of the trial participants, many with tumors that defied previous treatments, responded to the checkpoint inhibitor, says study leader Toni Choueiri, MD, clinical director of the Lank Center for Genitourinary Oncology. With colleagues Sabina Signoretti, MD, Eli Van Allen, MD, and others in the Harvard research community, he is analyzing preserved and fresh tumor tissue for biological signs that indicate which patients are likely to respond best to the treatment.

Meeting Patient Drives Home the Impact of Research

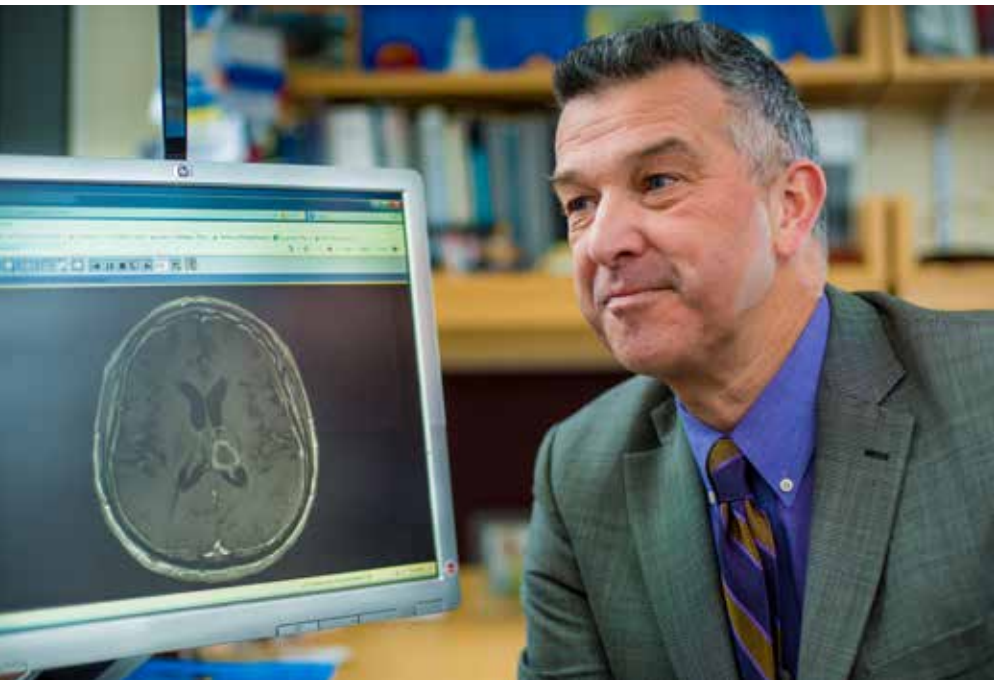
Radiation and chemo had done little to slow Barry Nelson's lung cancer when he started a clinical trial of an immune checkpoint inhibitor. But within a month, the disease was in retreat, with CT scans showing dramatic shrinkage of the tumors.

Nelson had been initially told he had only two years to live. Now, he was feeling so well, he started bicycling to his medical appointments. Dana-Farber nurse Joan Lucca, RN, MSN, asked if he'd like to meet the man who saved his life. A short time later, a gentle-mannered man walked up and introduced himself. It was Gordon Freeman, PhD, a scientist of nearly 30 years who leads the team whose discoveries led to the development of Nelson's treatment.

For a laboratory scientist such as Freeman, the opportunity to meet a patient who directly benefited from his work is exceedingly rare. Freeman had as many questions as Nelson did. "I asked him how it felt to get this new drug and when did he know that it was working?" Freeman says. "He's a real searcher; when the initial treatments failed, he wouldn't take no for an answer."

Nelson was struck by his easy rapport with Freeman. "He said it's marvelous to see that I'm doing so well, getting my health and my life back," Nelson says. "When my doctor showed him scans of how the tumors had shrunk, he wasn't just seeing a report, but a patient who had these results. It's been great to get to know the person who's given me this gift."

Researcher Gordon Freeman, PhD (right), meets with patient Barry Nelson.



David Reardon, MD, is leading clinical trials of a PD-1 blocker in patients with the brain cancer glioblastoma.

Lung Cancer

"Lung cancer is one of the cancer types where PD-1 blockers appear to be effective" – a surprise because previous immunotherapy approaches to the disease weren't successful – says Peter Hammerman, MD, PhD. He and his associates are studying the genetics of lung cancer to see if certain mutations render tumors more susceptible to immune checkpoint inhibitors. His colleague David Barbie, MD, is studying whether variations in the immune system from one person to another affect its cancer-fighting ability. The researchers also are exploring whether as-yet undiscovered checkpoint proteins play a role in holding off an immune system attack and could be new targets for immunotherapy.

Bladder Cancer

For some of the bladder cancer patients treated with a PD-L1 inhibitor in a recent phase 1 trial, the good news couldn't have come faster. At the first evaluation, six weeks after treatment began, there were already signs that the cancer was responding to it. After 12 weeks, there was tumor shrinkage in 52 percent of the patients whose infiltrating immune system cells had high levels of PD-L1 prior to treatment. Although more than half of participants experienced adverse side effects to the drug, known as MPDL3280A, none of them were particularly severe, says Joaquim Bellmunt, MD, PhD, who helped lead the trial. The drug has been designated a breakthrough therapy for bladder cancer by the FDA.

In early returns from clinical trials at other institutions, checkpoint inhibitors have shown good results in stomach cancer, head and neck cancers caused by the HPV virus, and some ovarian cancers, with less effectiveness in prostate cancer and colon cancer, Freeman notes. Much research remains, however, to determine where such agents are likely to have the biggest impact.

The future of immune checkpoint blockers for cancer almost certainly involves combination with other types of treatment – radiation therapy, targeted agents, cancer vaccines, and some chemotherapy agents – Freeman says. A recent study by Dana-Farber's F. Stephen Hodi, for example, found that patients with metastatic melanoma who were treated with ipilimumab survived 50 percent longer, on average, if they simultaneously received an immune system-stimulating agent. There's even evidence that radiation therapy works better when joined to treatment with checkpoint inhibitors.

More than a century after scientists recognized the immune system's potential as a cancer warrior, immunotherapy is rapidly becoming a mainstay of the anticancer arsenal.

Learn more about PD-1. Visit *www.dana-farber.org* and enter "freeman" in the search box.

KNOW YOUR SURROUNDINGS

BY ERIC BENDER

What help do tumors get from cells around them, and how can treatments stop that support?

ILLUSTRATION BY EVA TATCHEVA

Multiple myeloma is a poster child for recent advances in treatment: In the past decade, the U.S. Food and Drug Administration (FDA) approved no fewer than nine treatments for the blood cancer, and several more drug approvals appear to be near.

Not coincidentally, multiple myeloma is also a popular target that researchers use to study the interactions of tumor cells and their "tumor microenvironments" – the non-cancerous cells, molecules and blood vessels that surround and often support the malignant cells.

"These new myeloma drugs are all based on understanding how the tumor cells interact with other cells in their bone marrow environment," says Kenneth Anderson, MD, director of the Jerome Lipper Multiple Myeloma Center and LeBow Institute for Myeloma Therapeutics at Dana-Farber/Brigham and Women's Cancer Center (DF/BWCC).

"The whole idea is to reverse the way we think. Instead of treating patients when the disease has progressed, can we change the microenvironment to prevent progression?" – Irene Ghobrial, MD

Many labs studying numerous other cancers are also now targeting tumor microenvironments, hoping to replicate dramatic progress against multiple myeloma.

Managing Myeloma

Multiple myeloma begins with mutations in the development of B cells – white blood cells that can turn into plasma cells that normally fight bacteria or viruses. The first clinical sign is a condition called monoclonal gammopathy of undetermined significance (MGUS), with abnormal plasma cells appearing in the bone marrow. A small fraction of people with MGUS progress to smoldering myeloma, displaying higher populations of these cells. Most people with smoldering myeloma eventually develop active multiple myeloma, with low blood counts and increased risk of infection, kidney dysfunction, high blood calcium, and painful bone lesions.

Throughout the course of this disease, the interaction of these mutated plasma cells with their microenvironment is critically important.

"It's called cell-adhesion-mediated drug resistance, because binding of the tumor cell to the bone marrow triggers pathways mediating myeloma cell growth, survival and drug resistance," Anderson says. "The novel agents overcome the conventional drug resistance that's conferred by the microenvironment."

Numerous types of nearby cells may be enlisted to aid the tumor, and thus may be targets for new agents. One example is plasmacytoid dendritic cells (pDCs), which normally alert the immune system to respond to a suspicious cell.

"In myeloma these pDCs do not stimulate immune responses as they should, and even worse, they promote tumor cell growth and drug resistance," says Anderson. "So we are utilizing agents that target these pDC cells as potential myeloma therapies. In our models, we can use targeted treatments to mature these cells so they no longer promote tumor cells and acquire the ability to stimulate immune responses as they should. This result needs to be validated in clinical trials, but it's another proof-of-principle that the microenvironment really matters."

In another project reported in 2014, researchers led by Irene Ghobrial, MD, demonstrated that in mice, an agent that blocked a protein known as stromal cell-derived factor-1 (SDF-1), which is present in high levels in areas of the bone marrow with myeloma lesions, slowed the spread of the cancer and improved survival.

With this and many other research projects underway in her lab, "the whole idea is to reverse the way we think – instead of treating patients later on when the disease has progressed, can we change the microenvironment to prevent progression?" Ghobrial emphasizes. "We're designing trials now to take the best drugs we have and bring them earlier to smoldering disease."

Stromal Surroundings

Tumor microenvironments are studied broadly and deeply in other blood cancers, and in many solid tumors as well. "In breast cancer, there's a lot of data showing that the microenvironment is really important for tumor progression and for therapeutic resistance," says Kornelia Polyak, MD, PhD, a breast cancer geneticist at DF/BWCC.

Breast cancer cells are surrounded by a "stroma" composed mostly of fibroblasts (the most common connective tissue cell), blood vessels, and immune system cells. "The stroma frequently makes the cancer cells more resistant to treatment," says Polyak.

In one line of microenvironment research, Polyak's lab is focusing on the role of fibroblasts

from primary breast tumors and metastatic sites such as brain lesions that tend to be particularly resistant to treatment.

Other work examines the role of immune system cells in the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer. In normal breast tissue, these immune system cells go into the duct, but something stops them in DCIS so the tumor grows without interference from the immune system, she notes.

A third project looks at "myoepithelial" cells, which produce the barrier of connective tissue supporting breast ducts but tend to disappear as DCIS progresses.

Dana-Farber's Kenneth Anderson, MD (top), Irene Ghobrial, MD (lower left), and Kornelia Polyak MD, PhD (lower right), each explore different aspects of the tumor microenvironment – the molecules, cells, and blood vessels that surround and support a tumor.

Common Across Cancers

Despite all the obvious differences between breast cancer and multiple myeloma, researchers stress that the two diseases, and many other cancers, share many of the same molecular signaling pathways. "Learning about the biology from each other will be very significant," Anderson says.

One key theme across cancers is the role of the microenvironment in metastasis, the spreading of cancer to additional parts of the body. For example, tumor cells secrete "exosomes", tiny packages of protein and RNA that may help to seed metastases. Demonstrated in breast cancer, this phenomenon has been hypothesized to occur in myeloma as well. "Maybe that's why we start getting all those myeloma lesions growing fast at the same time," Ghobrial speculates.

Another common theme is the hope that drugs targeting the microenvironment may not need to be as selective as targeted drugs that seek out very specific mutations in tumor cells. Since the microenvironmental cells aren't mutated, each type can be attacked as a population and it can't develop resistance to drugs. "If you look at the successful treatments in myeloma, they are not mutation-specific," Ghobrial points out. For instance, proteasome inhibitors such as Velcade, which block the breakdown of proteins so that cells fill up with molecular garbage and die, "just kill any plasma cell," she says.

"If you think about treatments for myeloma or breast cancers or other tumors directed against intrinsic abnormalities in the cancer cell, that gives you a number of opportunities," Anderson sums up. "But if you target the host microenvironment or the interaction of the tumor with the microenvironment, you broadly expand the therapeutic options available."

Learn more online. Visit *www.dana-farber.org* and enter "microenvironment" in the search box.

SCIENCE ILLUSTRATED

BY ROBERT LEVY

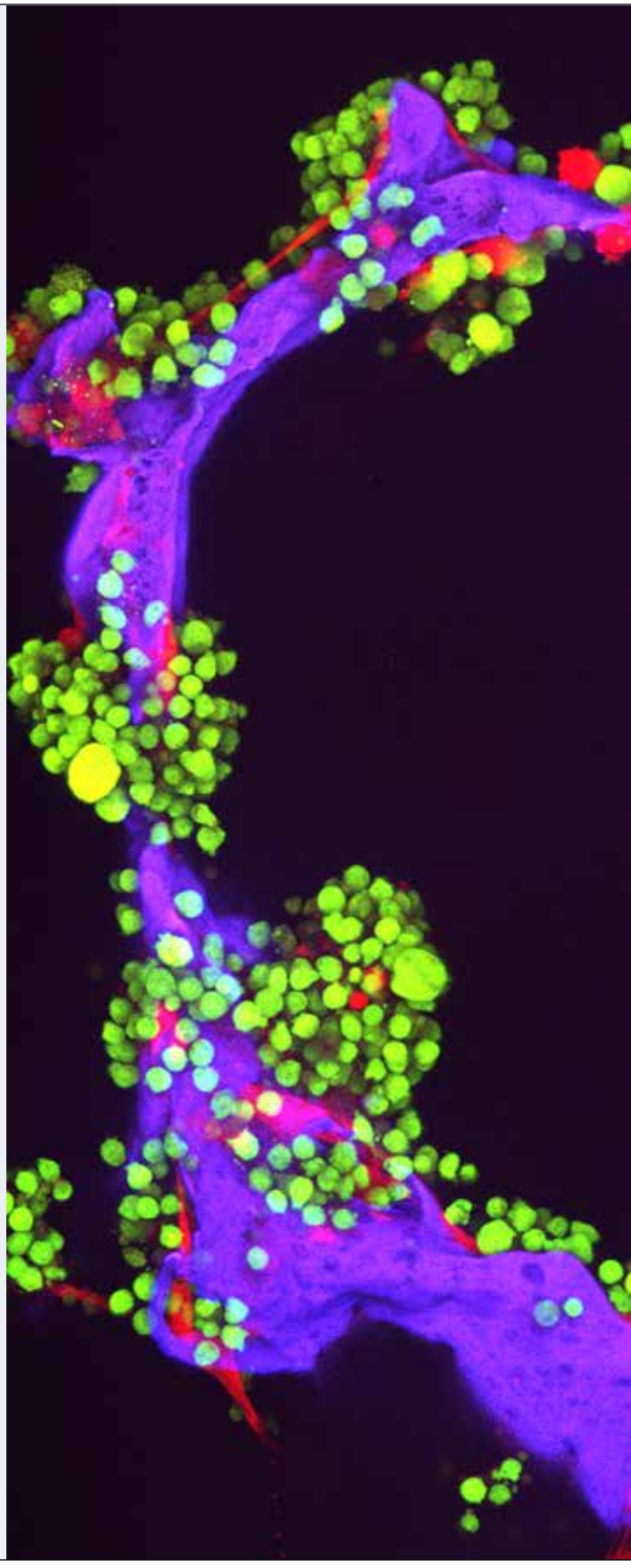
Master manipulators, cancer cells grow and survive by taking advantage of the normal, law-abiding cells around them. The traditional approach to studying cancer cells by growing them in laboratory dishes barren of anything but a broth of nutrients leaves many questions unanswered about how the cells interact with their surroundings.

To get a more realistic picture, researchers have developed laboratory models that simulate cancer's native, three-dimensional environment within the body. The image on these pages shows one approach. Made with a confocal microscope – which provides higher resolution than conventional light microscopes – the image shows multiple myeloma tumor cells (in green) and bone cells (red) growing on a scaffold made of silk protein (bluish purple).

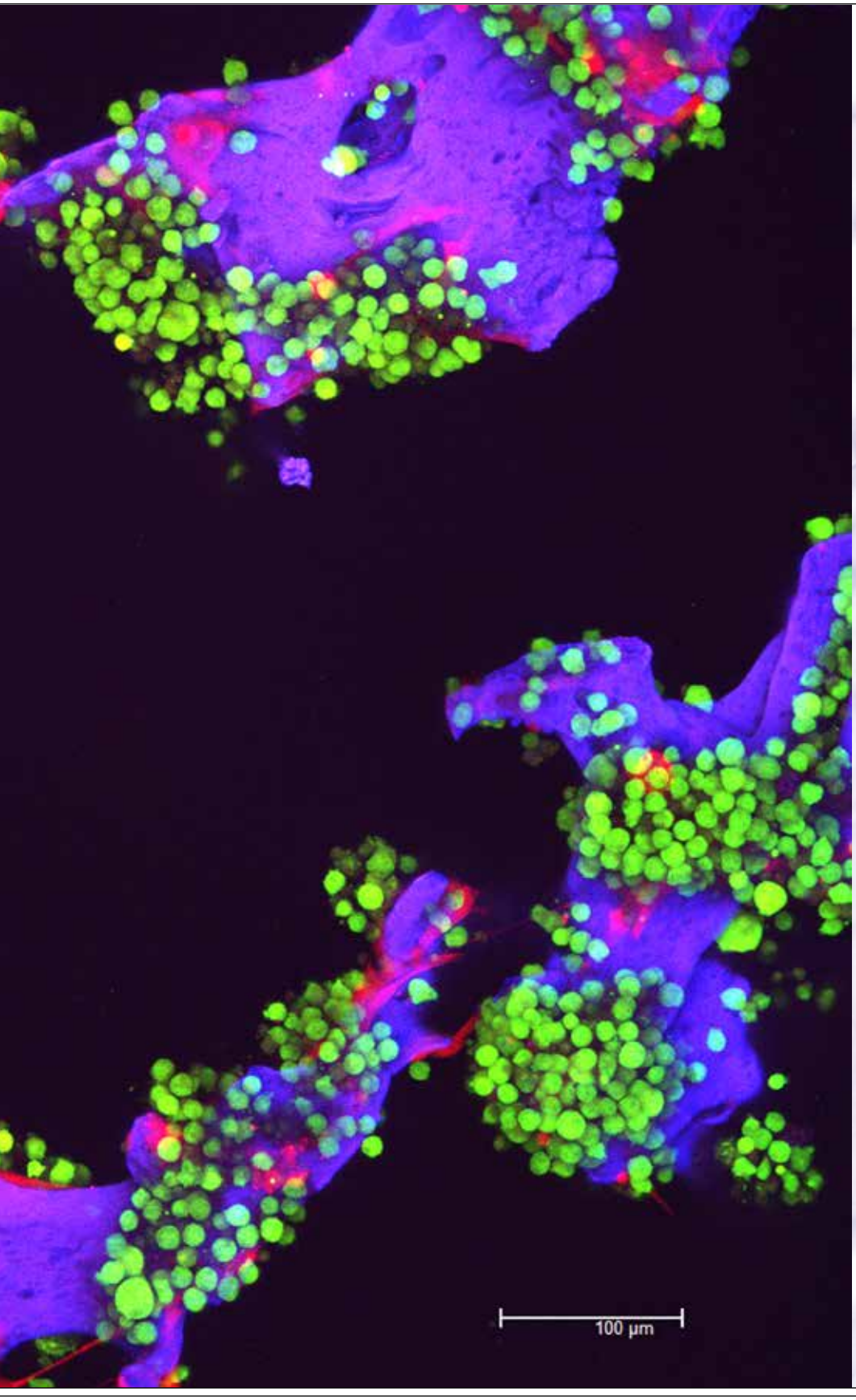
"The model is designed to resemble bone material, where myeloma cells grow, primarily," says Dana-Farber's Michaela Reagan, PhD, who studies the disease in the lab of Irene Ghobrial, MD. "We developed this silk scaffold-based, Tissue-Engineered Bone for cancer modeling with Dr. David Kaplan at Tufts University. We seed the scaffold with immature bone cells, or mesenchymal stromal cells, then add substances that cause these to develop into full-fledged bone cells, known as osteoblasts. This allows us to see how that process unfolds when myeloma cells are present and when they're absent."

Myeloma cells, it turns out, can prevent osteoblasts from maturing – and, by doing so, create a more hospitable environment for themselves. By comparing osteoblasts grown with myeloma cells from those grown without, Reagan and her colleagues have found differences that may yield clues to new treatments.

"Treatments that normalize the bone environment – that is, that allow pre-osteoblasts to resume their maturation into bone – may be effective in preventing myeloma from gaining a foothold in the body," Reagan says.



A MODEL HOME FOR MYELOMA



Family Ties

Why Genetics Matter

BY CHRISTINE HENSEL TRIANTOS

A family history of cancer prompted Sharon Goyette to undergo genetic testing at age 21.

On a cold winter day in 2002, Sharon Goyette stepped into Dana-Farber's Center for Cancer Genetics and Prevention. She was a 21-year-old college student, and this was the last place she wanted to be. But her mother had insisted.

After developing colon cancer, Goyette's mother had been diagnosed with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer), an inherited condition that increases the risk of many types of cancer, including colorectal, uterine, stomach, brain, and skin. Her colon cancer was now advanced, and she had pleaded with Goyette to undergo genetic testing to find out if she, too, carried the genetic mutation that would increase her own cancer risk – and, more importantly, take the necessary steps to avoid the same path.

So Goyette met with a genetic counselor and Sapna Syngal, MD, MPH, a gastroenterologist at Dana-Farber/Brigham and Women's Cancer Center who leads the Gastrointestinal Cancer Genetics and Prevention program. They explained the goal of the test was to keep Goyette healthy. If she didn't have the mutation, her risk was not elevated. If she did have the mutation, Syngal and her team would create a screening and prevention plan to reduce her risk of disease.

The results came back positive: Goyette had Lynch syndrome. "I was finishing school, trying to figure out what to do with my life, and on top of that I was losing my mom," she said. It was emotionally overwhelming, but she was reassured by the promise of continuing care provided by Syngal and her team.

Goyette is now 35, and has not been diagnosed with any cancer. Syngal continues to take an active role in Goyette's overall medical management, which includes a specially designed regimen of screenings and exams.

Who Should Consider Genetic Testing?

Genetic testing is on the rise. Amplified awareness, lower costs, and wider accessibility have prompted many people to wonder: Should I be tested?

Genetic testing isn't for everyone – at least not yet. Testing is recommended for people with personal or family histories that suggest an inherited genetic component. That could be a family pattern of cancer, or a close relative with a known gene mutation. It could also be someone diagnosed with cancer at an early age or with multiple cancer types.

Experts also recommend genetic testing for all women diagnosed with ovarian cancer, primary peritoneal cancer, or fallopian tube cancer, and for people who are diagnosed with rare tumors.

The reality is that more people should be tested than actually are, said Syngal.

"There's much more awareness now, but probably less than half of the people who should undergo testing are getting referred by their physicians," she said.

"Fear plays a role, and possibly misunderstanding of the implications," said Huma Q. Rana, MD, a geneticist and clinical director of the Center for Cancer Genetics and Prevention.

"People should not be afraid to discuss genetic testing with their doctors," said Judy Garber, MD, MPH, a medical oncologist and the longtime director of the center. "If their doctors have questions, we are here to help patients and their doctors figure it out."

In recent years, researchers have made significant strides in understanding cancer-related genetics. All cancers – including the hereditary forms – have accumulated mutations in their DNA that are responsible for their abnormal behaviors. However, five to 10 percent of all cancers are now attributed to inherited gene mutations. When these are identified, patients can use the information to guide screening and preventive behaviors that can help them to avoid the cancers that have occurred in their family members.

Sharon Goyette (right) meets annually with Sapna Syngal, MD, MPH (left), as part of her long-term management plan for an inherited genetic mutation.

Researchers fully expect to discover more gene mutations that increase the risk of various cancers. Currently, about 200 of those are recognized – including the two most commonly known, *BRCA1* and *BRCA2*.

"The field of genetics is changing very rapidly; there are almost daily advances," said Garber.

"People should not be afraid to discuss genetic testing with their doctors."

– Judy Garber, MD, MPH

The Process

Established in 1990, at what Garber noted was the "very beginning of hereditary cancer

"It's hard for people to make phone calls and say, 'I know we haven't talked in 30 years, but I must tell you we have a *BRCA* mutation in the family.'"

– Judy Garber, MD, MPH

genetics," the Center for Cancer Genetics and Prevention offers genetic testing and counseling for all of the syndromes known to be related to cancer risk. The center is staffed by medical oncologists, gastroenterologists, surgeons, genetic counselors, nurses, psychologists, a medical geneticist, and researchers.

"Other genetics centers have a 'diagnose and discharge' model," explained Rana. "Our center is unique because we continue to follow patients and their family members with these syndromes."

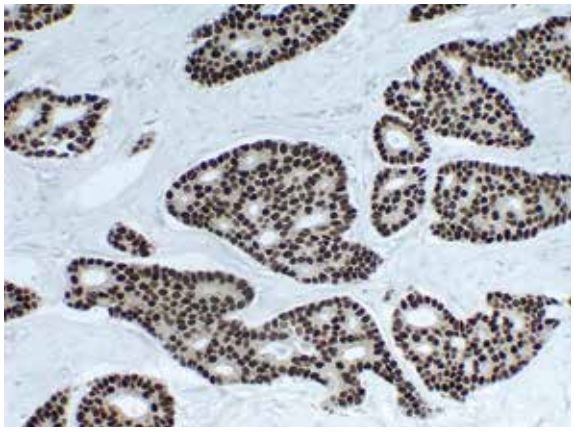
To assess genetic risk, it's important for people to understand and document their family health histories. "Patients really need to be proactive," said Syngal, who recommends appointing a family record keeper. "Families need to talk about their cancers and the particulars of their cancers as well as premalignant conditions." There are many types of premalignant conditions; examples include adenomatous polyps (colon), thyroid nodules, and sebaceous adenomas (skin).

The age at which people should be tested varies, said Rana. "In the case of a hereditary cancer syndrome that carries pediatric risk, we would recommend testing children." For breast syndromes and other syndromes that don't carry pediatric risk, testing typically starts at age 25, although testing for certain colon syndromes could start at 18.

Discovering you harbor an inherited mutation can carry an emotional burden, along with additional medical appointments and oversight, more frequent screenings, possible medications and prophylactic surgery, uncomfortable family discussions, and even potential challenges with life and disability insurance.

It's important, said Syngal, for patients to have guidance through this process. Before testing is done, genetic counselors meet with patients to discuss family histories as well as the risks and benefits of testing. They point out, for example, that patients might want to secure life and/or disability insurance before testing, since – unlike health insurance – there is no protection for people with pre-existing conditions or genetic risk. They discuss whether genetic testing makes sense, and outline the medical implications of a positive result.

Costs for genetic testing range considerably. For patients who are the first in their families to be tested, the average cost ranges from $1,500 to $3,500. If the testing is narrowed because a family genetic mutation has already been identified, the cost averages $300 to $400. Many private insurance plans will cover the cost,



Breast cancer cells.

but Medicare will cover costs for genetic testing only if the patient has already been diagnosed with cancer.

The test itself is swift and easy: a quick blood draw. (For people averse to needles, there's also a saliva-swab option.) Results are delivered in about a month.

If a patient is found to have an inherited mutation that increases cancer risk, physicians will develop a long-term management plan. Sharon Goyette's plan, for example, includes annual colonoscopies and dermatological exams as well as biennial upper endoscopies and uterine ultrasounds. At age 40, she will consider a prophylactic hysterectomy.

Family Considerations

Genetic testing results often have a ripple effect, spurring family interaction that can be both unifying and anxiety-producing.

"The minute there's a mutation identified in the family, we go through the family tree and identify anybody else who's at risk," explained Syngal. Often the patient will speak directly with those family members, but to allay any discomfort, the center can provide a letter with testing and screening recommendations that the patient can present to family members.

"We help people share the information," said Garber. "It's hard for people to make phone calls and say, 'I know we haven't talked in 30 years, but I must tell you we have a *BRCA* mutation in the family.' Sometimes patients ask us to talk with relatives directly to help educate them about the issues."

Huma Q. Rana, MD, helps patients understand what care may be needed now and in the future, including care for at-risk family members.

As someone with an inherited mutation, Goyette understands complicated family perspectives. "It's tricky," she said. "If you're related to someone with this diagnosis, it can be very sensitive to talk about it."

But she has never regretted her decision to be tested. "I'm glad I have this information and know how to be proactive," she said. "It's very empowering to know what you can do to keep yourself healthier."

Learn more online at *www.dana-farber.org/cancergenetics*

Solving Puzzles with Cigall Kadoch

BY RICHARD SALTUS

For researcher Cigall Kadoch, PhD, the Rubik's cube represents a love of puzzles, as well as the structure of the protein complexes she studies.

Growing up in the San Francisco area, Cigall Kadoch, PhD, had a passion for puzzles. The daughter of a Moroccan-born father and mother from Michigan who together developed an interior design business, Kadoch excelled in school and pretty much everything else. Above all, she loved to solve brain-teasers.

In high school, however, Kadoch came up against a problem that defied solution. Breast cancer took the life of a beloved family caretaker who had nurtured her interests in science and nature.

"I was deeply saddened and very angry at my lack of understanding of what had happened," recalls Kadoch, now a cancer researcher at Harvard Medical School (HMS) and Dana-Farber/Boston Children's Cancer and Blood Disorders Center.

At the time, she knew little about cancer except that it took lives far too early. "I thought to myself, cancer is a puzzle that isn't solved, let alone even well-defined, and I want to try. As naïve a statement as that was, it was a defining moment – one which I never could have predicted would actually shape my life's efforts."

Her sense of mission intensified after a summer at Harvard, where her uncle was a radiation oncologist. She spent time in his clinic, observing that some patients were cured and others worsened, and she wondered why. She found textbooks on biochemistry, oncology, and other fields fascinating. Then she was off to the races.

Kadoch blazed through college, taking pre-med courses, but she chose cancer biology for graduate study at Stanford University. "I couldn't drop this quest to solve puzzles and a chance to contribute new knowledge to the book of medicine," she explains. Dana-Farber Chief of Staff Stephen Sallan, MD, describes Kadoch as "addicted to discovery."

Working in the lab of prominent Stanford biologist Gerald Crabtree, MD, she authored two cancer research papers. The first, in the high-profile journal *Cell*, reported her discovery of how a gene mutation in a "chromatin-remodeling complex" leads to a rare, hard-to-treat cancer called synovial sarcoma. Its fundamental cause had been unknown. Remarkably, she and Crabtree were the only authors of the paper: an impressive achievement for someone so junior.

The second publication, in *Nature Genetics,* reported for the first time that at least 20 percent of all human cancers are driven, at least in part, by defects in one of the components of these chromatin complexes, called BAF complexes, that disrupt cells' orderly growth. With these insights, Kadoch broke new ground in the hot research area of cancer epigenetics – processes that change how genes operate without altering the genes themselves.

Hitting the Ground Running

In 2013, after earning her PhD in less than three years, Kadoch vaulted into a position in Dana-Farber/Boston Children's department of Pediatric Oncology, and is an assistant professor at HMS. At age 27, she was one of the youngest scientists ever appointed to the HMS faculty. She is also a member of the Broad Institute of Harvard and MIT.

George Demetri, MD, Dana-Farber's senior vice president for experimental therapeutics, played a major role in recruiting her. "Cigall came to Boston and gave a talk at the Broad Institute about her research. One of our investigators – James Bradner, MD – came back and said, 'We've got to get her here.'"

Stuart Orkin, MD, chair of Pediatric Oncology, noted that while some would find her youth and short track record as risks, "We were convinced she had the drive and the training and the smarts" to succeed.

In early 2014, she opened her Dana-Farber lab, stocking an empty laboratory space with equipment, mailing box after box of reagents from Stanford. As a new laboratory director, she is younger than several of the trainees she has brought on board.

Applying for "everything under the sun," she has funded her lab thus far through awards and grants, along with philanthropic gifts from donors who have heard her explain her research and its potential for fighting cancer in new ways.

In 2014, she received a $2.5 million innovator award from the National Institutes of Health and a $1 million American Cancer Society Research Scholar Award. Around the same time, she was named to *Forbes* magazine's list of "30 under 30" – the top people under age 30 who are making an impact on the world.

"She hit the ground running," observes Demetri. "She's focused like a laser and is just on fire with her work. The research is gaining wide recognition."

Chromatin and Cancer

Kadoch comes at the question of what causes cancer from an unusual direction. For decades, researchers focused on mutations and other alterations in the DNA of genes that force cells into uncontrolled, chaotic growth. Kadoch is interested less in how good genes go bad than in how mistakes in regulation of DNA structure cause normal genes to be activated at the wrong time, in the wrong place, or not activated when needed. And this process begins in the mechanism that stores our DNA and genes.

> "She's focused like a laser and is just on fire with her work."
>
> – George Demetri, MD

Some savvy travelers can cram several weeks' clothing into a carry-on bag. But for packing efficiency, it's hard to beat biology: Our entire human genome – a thin, 6-foot-long thread of DNA carrying about 20,000 genes – is squeezed into each cell's nucleus, a structure 1,000 times smaller than a pinhead.

Inside the nucleus, the DNA double helix is wound tightly around many spool-like protein structures – nucleosomes. This DNA-protein entity is called chromatin, which is both an organizational scaffold for DNA and the root of an intricate mechanism for switching genes on or off and regulating the translation of their blueprints into proteins.

Genes can't be turned on and made into proteins when chromatin is tightly coiled on its spools. So when the cell needs certain proteins to be made, the relevant spools unwind to expose a particular gene or genes to be read. Since these genes may be located at different sites along the chromosomes, the process requires different spools of DNA in various locations to unwind.

All of this chromatin shuffling must be tightly regulated so the right genes are turned on and off at the right time in the right place. This key task is performed by the chromatin-remodeling complexes. Each large complex is made up of multiple subunits that can assume various combinatorial assemblies, like the small individual blocks of a Rubik's Cube.

"The surprising thing we learned from recent sequencing studies is that these chromatin complexes play a significant role in cancer," Kadoch says.

Her team's research focuses on how malfunctions of a particular chromatin-regulatory complex called BAF can lead to the misregulation of genes that cause cancer. Normally, BAF complexes help protect the cell against cancer. However, structural mistakes in the subunits making up the chromatin regulatory complexes interfere with their ability to control gene expression, leading in certain situations to cancer. These structural flaws are caused by mutations in the genes that code for the subunit proteins.

Crabtree, her mentor at Stanford, had done elegant studies on how the BAF complex turns genes on and off to coordinate development of the vertebrate nervous system. But Kadoch was the first to show how abnormalities in the complex's structure could turn cells cancerous.

"We estimate that these mutations in chromatin complex subunits occur in more than 20 percent of human cancers," says Kadoch.

That doesn't mean that these mutations are the single driving force in all those cancers. But the research leading to her initial *Cell* paper was a tour de force that uncovered the mechanism behind the known lone culprit mutation and hallmark feature in synovial sarcoma.

"If we can understand how the flawed architecture of the chromatin complex can cause cancer, we can hopefully design specific therapeutic strategies," Kadoch explains. She and her team are using biochemical tools to reverse-engineer the structural makeup of the complexes.

"At this point we don't know how the puzzle pieces fit together in the complexes. We're asking how they are assembled in the first place. How do mutations affecting the complexes change which genes they go to and activate across the genome?"

Many targeted drugs block the activity of cancer-causing oncogenes. But it is notoriously difficult to treat cancer by restoring broken tumor suppressor proteins – like the BAF chromatin-regulatory complex that malfunctions to trigger synovial sarcoma and other cancers.

Kadoch remains undaunted.

Even if this particular strategy does not apply to other cancers caused by broken chromatin complexes, she believes that understanding how the faulty complexes trigger rare cancers will pay off in discovering the causes of more common cancers.

And that's a puzzle Kadoch is determined to solve.

Cigall Kadoch, PhD (left), heads a team of about 15 investigators at Dana-Farber.

Elena Togashi

Paying it Forward to Young Adult Patients

By SHANNON WATTERSON

Elena Togashi has worn many hats all over the world – finance expert in New York and Tokyo, negotiation consultant in Boston, and photographer in Vietnam, to name a few. But the experience that was most influential in leading her to Dana-Farber was her time as a young adult with cancer.

Togashi, who joined Dana-Farber in December 2013 as a business development manager in the Clinical Planning and Network Operations Department, was diagnosed with Hodgkin lymphoma at age 19, during her sophomore year at Brown University. The day was April 1, 1998. "I had never had surgery or been in the hospital in my life," says Togashi. "I sat there thinking, 'This is an April Fool's joke.'"

Togashi went through six months of chemotherapy and a month of daily radiation for her grapefruit-sized tumor, during which she lived with her family in New York City. "It was comforting to be at home, but when I was in treatment, all I cared about was getting back to college and being like my friends," she says.

Although her family and doctors were supportive, Togashi wasn't able to meet any other patients her own age during treatment and was instead focused on living as normal a life as possible.

Now that she's more ready to talk about her diagnosis, Togashi realizes she has few people with similar experiences to connect with. That's why, aside from her role adding new members to Dana-Farber's growing care network, Togashi is a part of the Institute's Young Adult Advisory Council. The group provides guidance to the Young Adult Program at Dana-Farber/Brigham and Women's Cancer Center, which supports patients ages 18-39 through individual counseling, online communities, and support programs and events.

"It's important to be able to talk to someone and know that your ideas aren't wrong, crazy, or inaccurate," Togashi says, adding that working at Dana-Farber has helped her process some of the lingering emotions from her own cancer experience. "I want college kids to feel like it's okay to talk about cancer."

Elena Togashi plays several roles at Dana-Farber.

"IT'S IMPORTANT TO BE ABLE TO TALK TO SOMEONE AND KNOW THAT YOUR IDEAS AREN'T WRONG, CRAZY, OR INACCURATE."

After finishing treatment, Togashi started participating in triathlons and marathons for Team-in-Training, which raises money for the Leukemia & Lymphoma Society. At the time of this writing, she's lacing up her shoes for the 2015 Boston Marathon as part of the Dana-Farber Marathon Challenge.

Although Togashi has a head for business, she hasn't given up on her dream of saving the world. Her role at Dana-Farber is a "perfect blend of my innate skills, what I really want to do, and feeling good about what I'm doing every day." As she helps to extend Dana-Farber's care to more patients, she's also able to help young patients meet other young adults and feel comfortable processing their emotions about cancer.

"I never would have guessed I'd be here, but now I can't imagine being anywhere else."

Tom Brokaw

As told to
SHANNON FITZGERALD

As a former "NBC Nightly News" anchor, Tom Brokaw experienced firsthand the most dramatic events of the late 20th century and today, but his life truly changed when he was diagnosed with multiple myeloma in August 2013.

"I've often wondered, as a journalist, how you react to those kinds of announcements about your life," Brokaw says. "I had no moment of panic; I had no moment of self-pity. My first thought was, 'The family was going to be okay... I'm going to have to find a way through this.'"

I couldn't have done it without my family. I couldn't have faced cancer without my wife, who was my pharmacist, sheriff, tough love, who laughed at my jokes, who got me out of bed, and was there for me. The family rallied around as well, which is also critically important.

Be sympathetic to those with cancer. I learned very quickly that the world was divided between those who have cancer, and have an entirely different view of it, and those who don't have it, and want to be sympathetic but have no full appreciation for what it's like to have cancer.

It's not math. There's a lot of sophisticated guesswork that goes on, like getting the right cocktail of drugs. I was not prepared for how uncertain it would be, and how there would be lows and highs along the way.

That patient-doctor relationship is critical. It becomes even more critical when you're dealing with something as mysterious as cancer. And it remains extraordinarily mysterious about how it begins, how you deal with it, how we're going to be able to get it under control at some point.

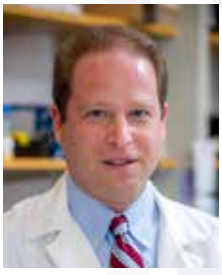
"I HAD NO MOMENT OF PANIC; I HAD NO MOMENT OF SELF-PITY. MY FIRST THOUGHT WAS, 'THE FAMILY WAS GOING TO BE OKAY... I'M GOING TO HAVE TO FIND A WAY THROUGH THIS.'"

Take inspiration from what [Dana-Farber] is doing. The astonishingly extraordinary success in the laboratories, and the people who are working to save lives – it's breathtaking. The research that is being done here and the commitment the community has to end of the war on cancer is really a big idea. It is a big idea that serves the general population well, it serves science well, and it serves future generations well. And that is a reward we can all enjoy and share in.

NBC News' Tom Brokaw shares his experience with cancer and offers advice to fellow patients.

Donna Berry, RN, PhD
Director, Phyllis F. Cantor Center for Research in Nursing and Patient Care Services

The question at the heart of my research is how to create opportunities (and an environment) for people with cancer to express themselves regarding risk and treatment issues and to participate fully as partners in their own care. This challenge led me to develop and study a decision support system for men with localized prostate cancer who face many different treatment options. Our team conducted a rigorous, national randomized trial of the Personal Patient Profile-Prostate (P3P), establishing the support system as the only efficacious system in the U.S. to meet the needs of a diverse population of men. The P3P provides an easy to use, Web-based support system that brings a man's personal preferences and factors to the decision table to be shared with the doctor's medical facts and recommendations



Loren Walensky, MD, PhD
Pediatric Hematology/Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center

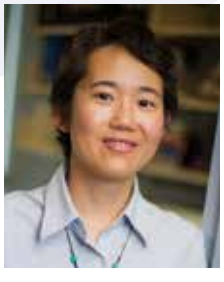
We can often make cancer go away when it is first diagnosed, but if it comes back, the challenge is more profound. How is it that nothing can stop the growth and survival of relapsed and refractory cancer cells?

My lab focuses on understanding, in exquisite molecular detail, the protein-protein handshakes that determine whether a cell lives or dies in response to stress or damage. The mechanisms of how "killer" and "survival" proteins turn on and off lie at the heart of cancer resistance. We've developed specialized chemical reagents that simulate the natural protein domains that control the death pathway. By deploying these reagents, we've discovered the location of the "on" switch for a major cellular killer protein, informing us how to develop new drugs to turn back on the death pathway in cancer cells.

Q&A

We asked three Dana-Farber researchers to share their insight on a common question:

What big questions are you seeking to answer in your research?



Xiaole Shirley Liu, PhD
Director, Center for Functional Cancer Epigenetics

As a genomics and computational biology laboratory, we develop statistical algorithms to help biologists interpret the massive amounts of data generated by genomics techniques, which explores the role of gene activity in normal development and disease. By statistically modeling the activity of thousands of genes within tumor cells, we aim to answer questions such as what genes go wrong in cancer, how are these genes regulated, what are the consequences of their malfunction, what are the therapies to target them, and what other therapies to use when tumors develop drug resistance.

Dana-Farber Cancer Institute

Founded in 1947 by Sidney Farber, MD, Dana-Farber Cancer Institute (*www.dana-farber.org*) is world renowned for its unique blend of basic and clinical research and for using its discoveries to improve treatments for cancer and related diseases. Consistently ranked one of the top cancer centers in the country by *U.S. News & World Report,* Dana-Farber is a founding member of the Dana-Farber/Harvard Cancer Center, which is one of 41 nationally designated Comprehensive Cancer Centers. As a teaching affiliate of Harvard Medical School, Dana-Farber is also one of 19 federal Centers for AIDS Research in the United States. It has earned "Magnet" status for excellence in nursing and is a QOPI® Certified Practice.

Dana-Farber partners with Brigham and Women's Hospital to deliver care for adults with cancer through Dana-Farber/Brigham and Women's Cancer Center. It also has a long-standing alliance with Boston Children's Hospital to care for pediatric cancer patients through Dana-Farber/Boston Children's Cancer and Blood Disorders Center. Bringing together the strengths of three world-class institutions, these partnerships provide an exceptional level of care for cancer patients and their families.

The Jimmy Fund

The Jimmy Fund (*www.JimmyFund.org*) solely supports Dana-Farber, raising funds for adult and pediatric cancer care and research to improve the chances of survival for cancer patients around the world. It is the official charity of the Massachusetts Chiefs of Police Association, the Pan-Massachusetts Challenge, and the Variety Children's Charity of New England. Since 1948, the generosity of millions of people has helped the Jimmy Fund save countless lives and reduce the burden of cancer for patients and families worldwide. Follow the Jimmy Fund on Facebook (*www.facebook.com/thejimmyfund*) and on Twitter (*@TheJimmyFund*).

10% of all designated gifts supports our Faculty Research Fund to advance Dana-Farber's research mission.

DANA-FARBER CANCER INSTITUTE
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Paths of Progress Spring/Summer 2015

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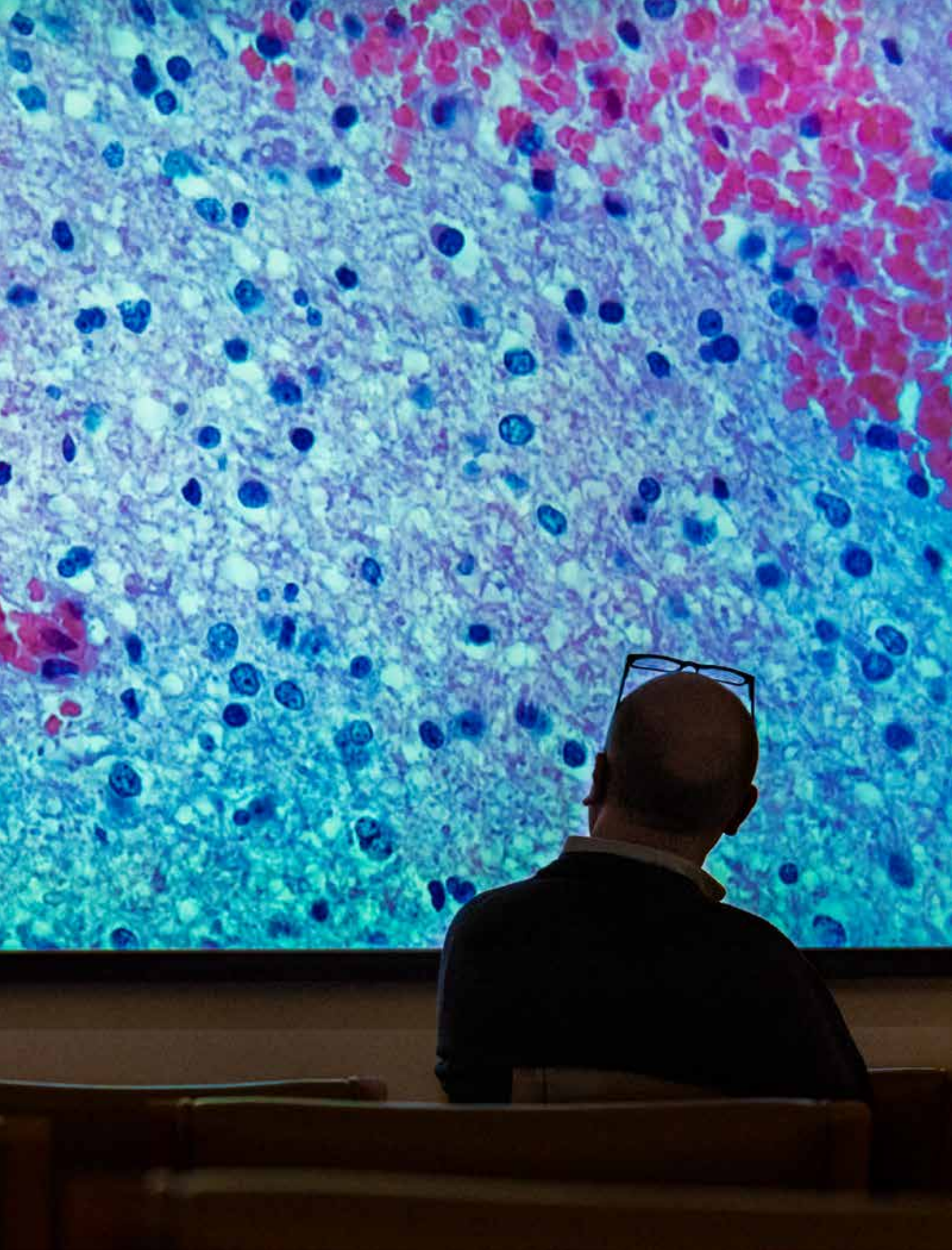


A message from Chief Financial Officer Karen Bird

Dana Farber's strong financial performance in fiscal year 2014 culminated in an increase in net assets of approximately $130 million, during a year of continued investment in our physical plant, research, and IT systems. We achieved an operating margin of $21 million, or roughly 2.0 percent, compared to a budgeted gain of $16 million. Non-operating revenue also had strong performance, resulting in an excess of revenues over expenses of $32.1 million. Total cash and investments grew 17.6 percent, due to strong operating and fundraising activity and a 9.6 percent return on our portfolio for the 12-month period ended Sept. 30, 2014.

Patient care revenue increased by 6.9 percent at the main Boston campus and our regional satellite centers, continuing our trend of the last several years. Thanks to the ongoing support of our donors, it was a robust year for fundraising, which saw a 1.5 percent increase in unrestricted giving. Research revenues remained fairly constant between 2013 and 2014, increasing 1.0 percent. This rate of growth is similar to last year and reflects the continued impact of the reductions in funds available for research from the National Institutes of Health (NIH). Our researchers compete very effectively for available NIH funding and our strong sponsored research growth has offset reductions in this funding.

Our investments in research and clinical care in 2014 position us well for the future. Most notably in research, our financial investment in the opening of the Longwood Center is complete and investigators and their staff began moving into this state-of-the-art laboratory facility in January 2015. The ongoing costs of occupying the Longwood Center and the recruitment of related faculty begin in 2015 and reflect our commitment to best-in-class scientific advancements.

On the clinical side, we expanded our regional network by opening a satellite at St. Elizabeth's Medical Center in Boston. The new satellite marks the first collaboration with Steward Health Care, one of the largest community networks in Massachusetts. In addition, we purchased a 15-physician medical oncology practice, our flagship Dana-Farber Community Cancer Care (DFCCC) physician group (previously Commonwealth Hematology Oncology). DFCCC is an exciting opportunity to provide community oncology in a physician practice setting.

In 2014, a great deal of time and resources went into preparation for our transition to a new clinical and revenue software platform from Epic Systems. This new set of applications will enhance safety features, allow critical access to data, and bring efficiencies in our work. We will move to the system in May 2015.

Management, faculty, and staff throughout Dana-Farber – guided by the oversight of several committees of our Board of Trustees – worked diligently to achieve these results. We are grateful to them and also to the many donors and friends of Dana-Farber, who continue to demonstrate their commitment to the organization with their valuable knowledge and generous contributions. We are proud of all of these efforts and thankful for this strong and ongoing support.

CONDENSED CONSOLIDATED BALANCE SHEETS

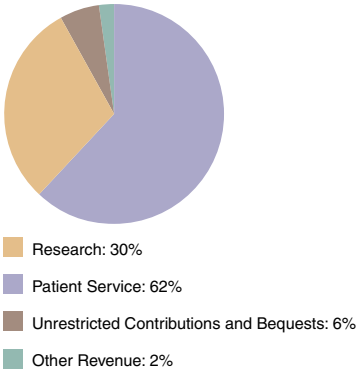
For the Fiscal Year Ended Sept. 30	2014	2013
(Dollars in thousands)		
Assets		
Current Assets	$233,941	$311,537
Investments	948,680	747,048
Debt Service Reserve and Construction Fund	12,586	12,544
Property, Plant, and Equipment, net	694,132	646,077
Contributions Receivable, less current portion	37,748	49,432
Other Assets	23,571	23,790
Total Assets	**$1,950,658**	**$1,790,428**
Liabilities and Net Assets		
Current Liabilities	$222,743	$193,306
Long-Term Debt and Other Liabilities	415,765	414,739
Net Assets		
Unrestricted	606,863	556,219
Temporarily Restricted	538,070	467,303
Permanently Restricted	167,217	158,861
Subtotal Net Assets	1,312,150	1,182,383
Total Liabilities and Net Assets	**$1,950,658**	**$1,790,428**
Summary Statistical Information		
(unless otherwise noted, includes adult and pediatric patients)		
Infusion Treatments	131,017	127,031
Outpatient MD Visits	252,582	235,254
Number of Licensed Beds (as of year-end)	30	30
Adult Inpatient Discharges	1,059	990
Clinical Trials (open to patients at Dana-Farber, including therapeutic and nontherapeutic trials)	761	767

** Subsidiaries include Dana-Farber Inc., Dana-Farber Cancer Care Network, and Dana-Farber Trust.*

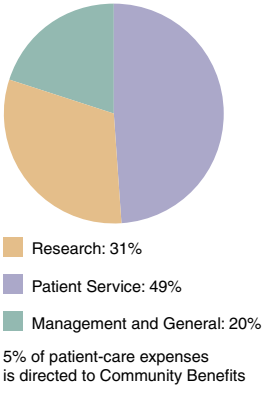
Condensed Consolidated Statements of Operations and Changes in Net Assets

For the Fiscal Year Ended Sept. 30 *(Dollars in thousands)*	2014	2013
Revenues		
Research	$322,101	$318,815
Patient Service, net	679,175	635,458
Unrestricted Contributions and Bequests	61,183	60,255
Other Operating	24,179	19,255
Total Revenues	**$1,086,638**	**$1,033,783**
Expenses		
Direct Research	280,130	272,968
Direct Patient Care	441,875	417,026
Indirect	343,379	324,758
Total Operating Expenses	**$1,065,384**	**$1,014,752**
Operating Income	21,254	19,031
Investment Return, net	22,028	23,951
Interest Rate Swap Agreement		
Net interest received/(paid)	(5,611)	(5,569)
Change in fair value	(5,615)	18,764
Excess of Revenues Over Expenses	32,056	56,177
Other	18,588	22,817
Increase in Temporarily Restricted Net Assets	70,767	60,364
Increase in Permanently Restricted Net Assets	8,356	6,862
Increase in Net Assets	129,767	146,220
Net Assets at Beginning of Year	1,182,383	1,036,163
Net Assets at End of Year	**$1,312,150**	**$1,182,383**

Fiscal Year 2014 Income

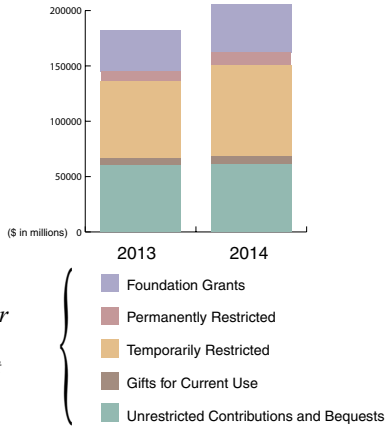


Fiscal Year 2014 Expenses



5% of patient-care expenses
is directed to Community Benefits

Comparison of Private Support



The preceding selected consolidated financial data as of Sept. 30, 2014, and 2013 (except for the summary statistical data) have been derived from the consolidated financial statements of Dana-Farber Cancer Institute Inc., Dana-Farber Inc., Dana-Farber Cancer Care Network, and Dana-Farber Trust. These have been audited by Ernst & Young, LLP, independent auditors.

In FY 2014, the Institute raised $230 million in new gifts and new pledges through its Division of Development and the Jimmy Fund, and through the Friends of Dana-Farber Cancer Institute. For accounting purposes, the financial charts reflect new gifts and new pledges calculated at present value, excluding commitments the Institute could not record due to conditionality.

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Navigating the bone marrow niche: translational insights and cancer-driven dysfunction

Michaela R. Reagan and Clifford J. Rosen

Abstract | The bone marrow niche consists of stem and progenitor cells destined to become mature cells such as haematopoietic elements, osteoblasts or adipocytes. Marrow cells, influenced by endocrine, paracrine and autocrine factors, ultimately function as a unit to regulate bone remodelling and haematopoiesis. Current evidence highlights that the bone marrow niche is not merely an anatomic compartment; rather, it integrates the physiology of two distinct organ systems, the skeleton and the marrow. The niche has a hypoxic microenvironment that maintains quiescent haematopoietic stem cells (HSCs) and supports glycolytic metabolism. In response to biochemical cues and under the influence of neural, hormonal, and biochemical factors, marrow stromal elements, such as mesenchymal stromal cells (MSCs), differentiate into mature, functioning cells. However, disruption of the niche can affect cellular differentiation, resulting in disorders ranging from osteoporosis to malignancy. In this Review, we propose that the niche reflects the vitality of two tissues — bone and blood — by providing a unique environment for stem and stromal cells to flourish while simultaneously preventing disproportionate proliferation, malignant transformation or loss of the multipotent progenitors required for healing, functional immunity and growth throughout an organism's lifetime. Through a fuller understanding of the complexity of the niche in physiologic and pathologic states, the successful development of more-effective therapeutic approaches to target the niche and its cellular components for the treatment of rheumatic, endocrine, neoplastic and metabolic diseases becomes achievable.

Bone marrow is a remarkable multifunctional tissue that contains stem, progenitor and mature cells of several lineages. Stem cells have, by definition, the capacity to self-renew and differentiate into many different types of cells[1] (FIG. 1). Haematopoietic stem cells (HSCs) are multipotent cells that differentiate into myeloid, lymphoid and erythroid lineages, and have short-term or long-term regenerative capacity. By contrast, bone marrow cells of purely mesenchymal origin — that is, mesenchymal stromal cells (MSCs) — considered within a strict definition based on cell-surface markers and function (TABLE 1), include cells capable of tissue culture plastic adherence and expansion but exclude the non-stromal elements of the marrow such as osteoclasts, macrophages, and endothelial cells[2]. The bone marrow 'stem cell niche' refers to the unique microenvironment of these regenerative cells in the bone marrow. This niche forms an anatomical and functional unit of physiology that integrates endocrine, autocrine, and paracrine signalling to serve the needs of the whole organism by sustaining the stem cell pool[3].

Mobilization and export of HSCs from the bone marrow can occur with injury, acute inflammation or biochemical stress, or during tissue repair. The stem cell pool that remains in the bone marrow after stress-induced haematopoiesis is essential for its repopulation and revitalization. In this Review, we summarize the unique physiological aspects of the bone marrow niche and the consequences of its alteration. In addition, we examine this microenvironment as it relates to cancer cells that invade and hijack the function of the niche[4–6].

Function and anatomy of the niche

Functional aspects

The bone marrow niche has several functions including the production of blood-forming elements, the maintenance of HSCs, and the modulation of skeletal remodelling. HSCs have the unique capacity to give rise to all mature blood cell types and are self-renewing; that is, during asymmetric division a proportion of the daughter cells remain HSCs, such that the pool of these cells is not

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Key points

- The bone marrow niche supports the integration of two major organ systems — the skeleton and the marrow
- The niche is a unique microenvironment that is crucial for haematopoietic stem cell quiescence
- Important features of the niche include its cellular components, hypoxia, extracellular matrices, cytokines and growth factors, and vascularization
- Multiple myeloma and other cancer cells hijack and alter the bone marrow niche, and are altered by the niche in turn; thus, targeting niche–cancer interactions is a promising therapeutic avenue
- Novel *in vitro* and *in vivo* models of the bone marrow niche and cancer cells enable us to better understand interactions between cancer and bone marrow niche cells
- A more complete understanding of the biology of the unique bone marrow microenvironment must remain a major research priority

depleted. Within the HSC pool are transient self-renewing HSCs and long-term quiescent HSCs. By contrast, bone marrow MSCs that regulate bone remodelling are multipotent, self-renewing progenitor cells that can differentiate into other cell types (for example, osteoblasts, adipocytes or chondrocytes)[7]. HSC quiescence is strongly dependent on MSCs and MSC-descendants, illustrating an important role for MSCs in the bone marrow niche[8].

The delicate balance between HSCs and MSCs provides a critical integration point between the marrow and the skeleton. In physiologic states, as well as in injury and chronic diseases, several types of progenitor cells are required to perform specific functions beyond bone remodelling and maintenance of haematopoiesis. Importantly, roles for the niche in modulating tumour biology and immunity have also emerged, further enhancing its importance but also emphasizing its complexity.

Location and organization

Defining the physical location of the bone marrow niche can be challenging because of considerable inter-individual and inter-species differences in the bone marrow. Classically, the principal bone marrow niche component is that which supports the cells that ultimately defines haematopoiesis; that is, HSCs, which are destined for differentiation and subsequent export into the circulation[9]. Today the bone marrow niche is reconsidered within the functional context of MSCs, which are essential for maintaining a stable pool of osteoblast progenitors for bone remodelling[10,11]. Human and rodent MSCs have been used experimentally as therapeutic tools to hasten fracture repair or injury recovery in many tissues[11,12]. Enhanced MSC differentiation into osteogenic lineage cells has been directly linked to the maintenance of HSCs in their quiescent state[13]. Still, the balance between quiescence, self-renewal, and differentiation of HSCs in the bone marrow niche is dynamic, and modulated in part by MSCs. Hence, what has emerged is a new concept of dual

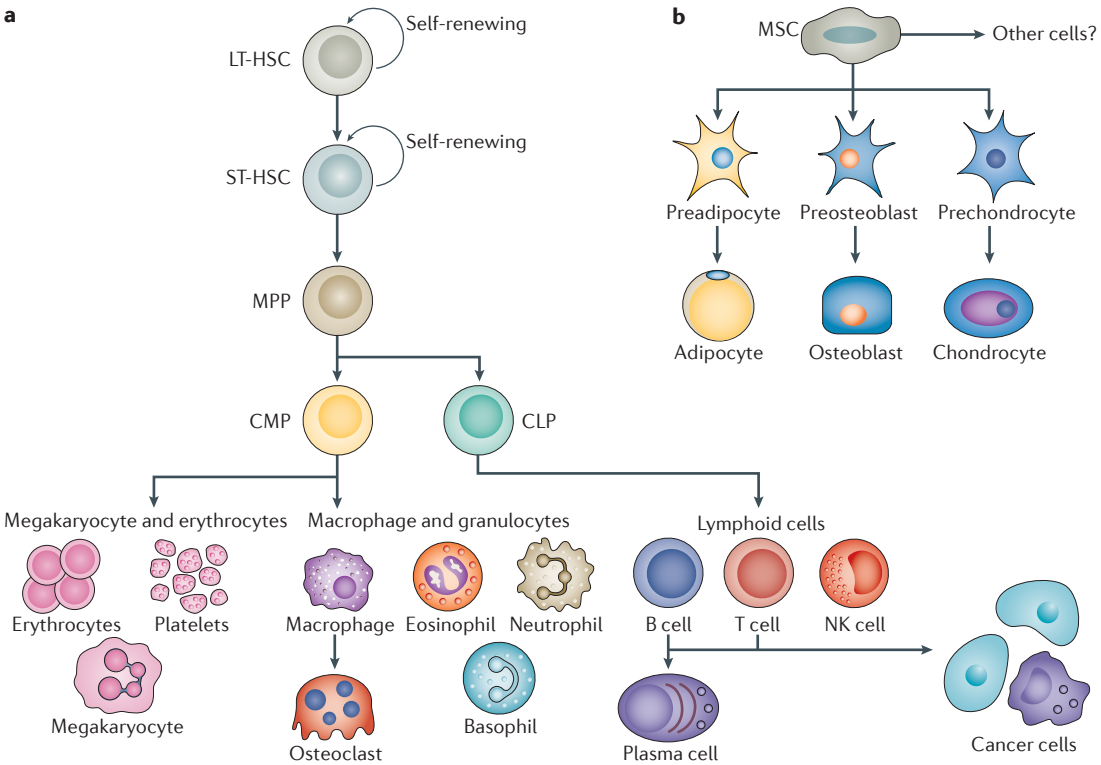

Figure 1 | **The regenerative cells of the bone marrow niche. a** | Haematopoietic stem cells (HSCs) give rise to all mature blood cell types. A proportion of these self-renewing cells remain stem cells to maintain a pool of long-term reconstituting HSCs (LT-HSCs) and short-term reconstituting HSCs (ST-HSCs). **b** | Bone marrow mesenchymal stromal cells (MSCs) are multipotent, self-renewing progenitor cells that can differentiate into other cell types. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MPP, multipotent progenitor; NK cell, natural killer cell.

Table 1 | **Main components of the bone marrow niche**[7,11,17,179–181]

Cell type	Location	Proximity	Human surface marker(s)	Effect of low O_2 %	Metabolism
HSC	Endosteal surface	Sinusoids	Lin^-CD34^+,$CD59^+$, $CD90/Thy1^+$, $CD38^-$ $CD45RA^-$	Stemness	Glycolysis
MSC	Endosteal surface, blood vessels and marrow space	Sinusoids, endothelial cells	Lin^-, $CD31^-$, $CD34^-$, $CD45^-$ $Stro1^+$, $CD\ 105^+$, $CD106^+$, $CD146^+$, $CD271^+$, ALP^+	Differentiation	Glycolysis
Bone lining cell	Endosteal surface	Osteoblasts	None known	Not known	Not known
Osteomac	Endosteum, diaphysis	Perivascular region	$CD68^+$ ($F4/80^+$ in mice)	Not known	Glycolysis (?)
Bone marrow adipocyte	Adjacent to the endosteal surface in the metaphysis	Osteoblasts, HSCs	Perilipin	Not known	Not known

HSC, haematopoietic stem cell; MSC, mesenchymal stromal cell.

stem cell populations that share a common location, with an interdependent relationship of both mutual support and competition for the marrow space[14].

Anatomically, the niche is more than a series of HSCs clinging to MSCs and osteoprogenitors within a flat marrow space. Rather, it is a 3D structure adjacent to the perivascular (or sinusoidal) space created by endothelial cells, in close proximity to trabecular bone and other cells[15,16]. In addition to those components, the niche also has matrix elements and microvessels that probably have regulatory capacities, particularly with respect to the unique biochemical composition of the milieu (see later). In that vein, the niche can be considered as two mini organ systems interacting at a single site that has specific environmental characteristics[17] (FIG. 2). Notwithstanding this concept, reconciling the role of trabecular bone with haematopoiesis remains a challenge. However, the identification by Bianco, Robey and others of the cellular phenotype of multipotent MSCs ($CD146^+$ adventitial cells adjacent to sinusoids) and emerging evidence of the role of bone lining cells has allowed for a more-complete understanding of the organization of the haematopoietic niche, and the importance of MSCs and the endosteal surface of trabecular bone to the production of differentiated marrow elements[7,18,19].

Cellular components

As well as HSCs and MSCs, several other cellular components of the bone marrow have been recognized as critical for the maintenance of a healthy niche. The aforementioned cells include bone lining cells, osteoblasts, marrow adipocytes, resident tissue macrophages ('osteomacs'), immune cells, canopy cells, and neurons, which are discussed respectively in this section; features of the main cellular components of the bone marrow niche are summarized in TABLE 1. These cells are simultaneously reliant on the niche and essential for its function.

Bone lining cells on the endosteal surface that express transcription factor Sp7 (also known as osterix) are precursors to osteoblasts and osteocytes[20]. These cells have been difficult to characterize but generally are flat and fibroblastic in appearance. They could have an important role in replacing bone-forming cells during physiologic remodelling and in response to anabolic therapies such as parathyroid hormone (PTH)[21]. Whether these cells can also differentiate into adipocytes remains to be proven but raises the interesting concept of a secondary pool of multipotent MSCs within the niche proper.

Osteoblasts are critical regulators of HSC fate within the bone marrow niche[22]. These cells are in close proximity to HSCs; moreover, regulatory factors that enhance MSC differentiation into the osteoblastic lineage can also promote the development of the bone marrow niche as well as the expansion and egress of HSCs[23]. The Kronenberg group was one of the first to establish the importance of osteoblasts in maintaining HSCs within the niche[13]. The same group also showed that recombinant PTH stimulated expansion and egress of HSCs, and that constitutive upregulation of the PTH–PTH-related peptide (PTHrP) receptor in osteoblasts disrupted normal haematopoiesis[24]. Ten years later, Coskun and colleagues showed that lack of Sp7 in osteoblast-lineage cells allowed for vascularization of developing bone marrow, but HSC proliferation and differentiation potential were considerably impaired[25]. In addition, Omatsu *et al.*[26] reported that expression of the transcription factor FoxC1 in osteoprogenitor and CXCL12-abundant reticular (CAR) cells was essential for maintenance of haematopoietic stem and progenitor cells *in vivo*. Thus, MSCs, osteoblasts and their progenitors are essential components for niche homeostasis.

Marrow adipocytes, which reside close to the endosteal surface, were long considered 'filler' for the marrow compartment. This notion was based on anatomical studies of marrow from patients with age-related osteoporosis, aplastic anaemia, myelodysplasia, and after radiation or chemotherapy[27], in which adipocytes were abundant within fibrotic or empty marrow spaces[28,29]. However, work in the past 3 years has demonstrated a much more complicated role for the adipocyte within the bone marrow niche[30]. For example, there are probably two major types of marrow adipose tissue (MAT): one that is found in the distal tibia and tail of rodents and which is formed shortly after birth (constitutive MAT), and one that is noted later in life in the proximal femur and vertebrae in close proximity

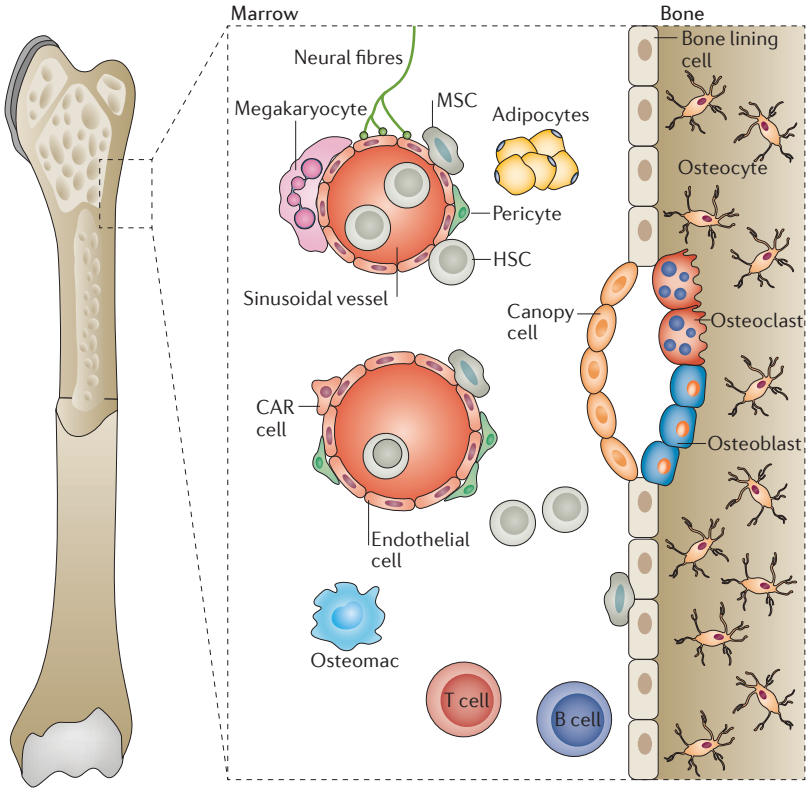

Figure 2 | **The two mini-organs of the bone marrow niche.** The bone marrow niche is composed of multiple cells of mesenchymal and haematopoietic lineages. A hypoxic environment, endosteal bone cells and the proximity of sinusoids and microvessels provide a unique environment for haematopoietic stem cells (HSCs) and mesenchymal stromal cells (MSCs). The union of the skeletal remodelling system and the vascular network within the bone marrow provides a unique niche that regulates whole-body homeostasis. CAR cell, CXCL12-abundant reticular cell.

to haematopoietic elements and trabecular bone (regulated MAT)[31]. Constitutive MAT negatively regulates haematopoiesis, possibly by maintaining HSCs in a quiescent state, as shown in elegant experiments by the Daley group[32]. With regard to the skeleton, constitutive MAT volume, as measured by use of osmium staining and microCT in rodents and MRI in humans, is inversely related to bone mass[33,34]. By contrast, regulated MAT seems to be a more dynamic adipose tissue that has endocrine and paracrine effects on both the haematopoietic and skeletal remodelling systems[35]. A high volume of regulated MAT is often, although not always, associated with low bone mass[36,37].

Osteomacs (bone-marrow-resident macrophages; F4/80$^+$ in mice, CD68$^+$ in humans) are also pivotal to the maintenance of the endosteal bone marrow niche[38]. Osteomacs facilitate the homing to bone, colonization, and dormancy of HSCs, and loss of osteomacs leads to the egress of HSCs into the blood[39]. Osteomacs are distributed throughout the endosteum and periosteum and are vital to MSC osteogenic differentiation *in vivo* and *in vitro*[38,40], potentially contributing to the canopy covering the bone remodelling canopy (discussed later) in bone modelling sites[41]. Winkler *et al.*[39] suggest that osteomacs might also maintain haematopoietic progenitors in their stem-cell state while supporting endosteal bone formation. In support of that tenet, treatment with granulocyte colony-stimulating factor motivates HSCs to differentiate while at the same time depleting endosteal osteoblast and osteomac populations[39].

Immune cells are an additional component of the bone marrow niche. Macrophages, neutrophils and myeloid-derived suppressor cells synergize to preserve the unique environment of the niche and protect it from cytotoxic lymphocytes[42]. These innate immune cells are stress-responsive and, thus, can communicate with other cells within the niche to regulate HSC egress. In essence, the presence of this immunomodulatory network establishes a permissive environment that could enable colonization by foreign cells. This is sometimes referred to as 'bone-specific immunity', as the niche is an immune-privileged environment[43]. The consequences of this specialized milieu are evident clinically in the frequent occurrence of tumour metastases in bone (discussed later), as well as serious infections such as tuberculosis involving this tissue before widespread dissemination[44].

A 'canopy' or cellular membrane has been described that covers not only cuboidal osteoblasts in the periosteum but also the bone remodelling unit[45,46]. This canopy might serve several functions beyond the insulation of bone cells from external stimuli or invasion. It might also be an important part of the niche, as it is composed of flattened cells and microvessels that provide nutrients and substrates for cells contained within that space, including MSCs, bone lining cells, osteoblasts, osteoclasts, and osteocytes[45,46]. The precise relationship between the canopy and the niche has not been determined, in part because of the difficulty in defining both their anatomic locations and the lack of functional tools to trace canopy cell activity.

Three other key cell types that contribute to the niche are endothelial cells, pericytes and megakaryocytes. Megakaryocytes contribute profoundly to the niche by supporting HSC quiescence through production of platelet factor 4 (also known as CXCL4) and transforming growth factor (TGF)-β[47,48]. Sinusoidal endothelial cells, through expression of E-selectin[49], stromal cell-derived factor (SDF1, also known as CXCL12 (REF. 50)) and vascular endothelial growth factor (VEGF) receptor 2 (REF. 51), are critical for HSC maintenance and haematopoietic recovery from myeloablation. Importantly, pericytes, which enwrap endothelial cells of vessels, were found to be necessary for HSC homeostasis primarily through expression of stem cell factor by perivascular cells throughout the bone marrow[52].

Finally, neural regulation of the niche and control of HSC export is mediated primarily through the sympathetic nervous system (SNS). The cyclic release of HSCs and expression of SDF1 and its receptor, CXCR4, are regulated by molecular clock genes, mediated by circadian secretion of noradrenaline from sympathetic nerves[53]. These adrenergic signals are delivered locally by nerves in the bone marrow and transmitted to MSCs through β3-adrenoceptors. Moreover, MSCs express α1B, α2A,

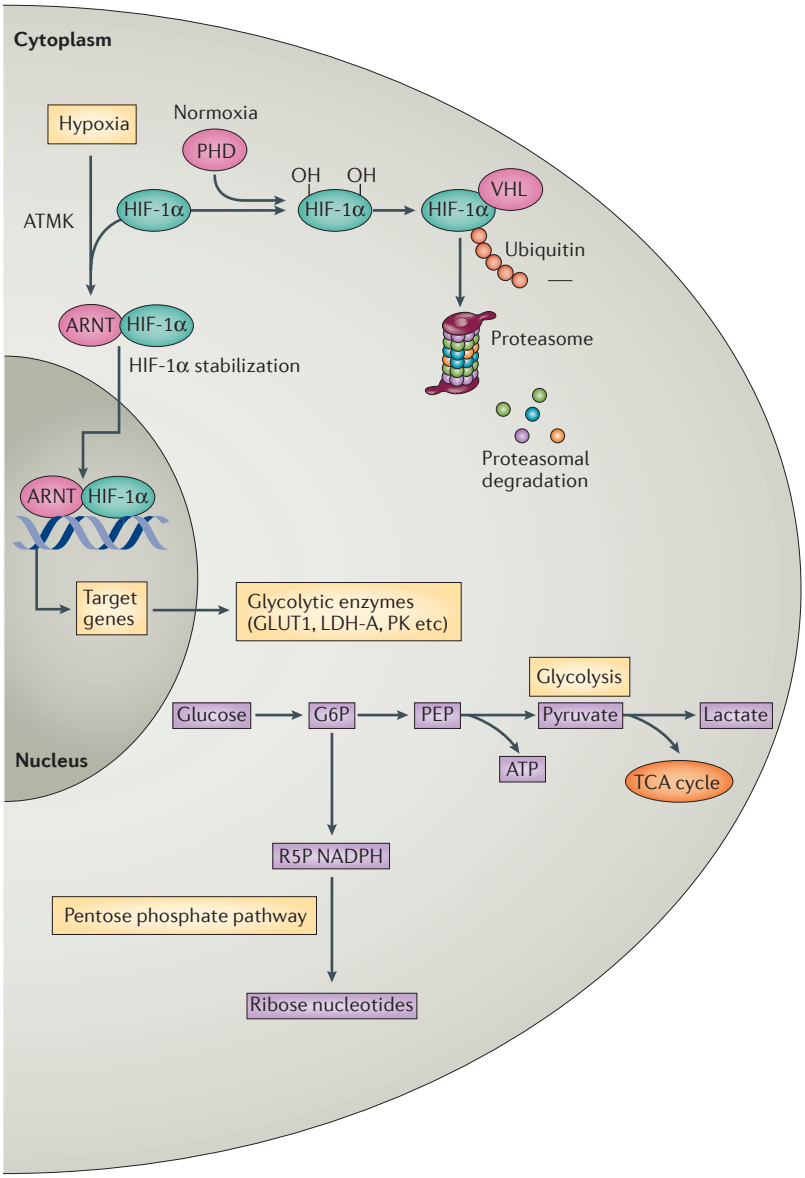

Figure 3 | **Biochemistry of the bone marrow niche.** The hypoxic environment of the niche leads to changes in substrate utilization in order to fuel cellular activity. Hypoxia induces ATM kinase (ATMK), which catalyses hypoxia inducible factor-1α (HIF-1α) activity. HIF-1α is stabilized by binding to aryl hydrocarbon receptor nuclear translocator (ARNT); the HIF-1α complex enters the nucleus and regulates a number of genes, particularly those related to glycolysis. Consequently, glucose is utilized for ATP generation through lactate production and via the pentose phosphate shunt for nucleotide synthesis that is essential for cell proliferation. Alternatively, and particularly during normoxia, HIF-1α is rapidly broken down in the proteasome via ubiquitination by proline hydroxylase domain (PHD) enzymes. G6P, glucose-6-phosphate; GLUT1, glucose transporter 1; LDH-A, lactate dehydrogenase A; PEP, phosphoenolpyruvate; PK, protein kinase; R5P, ribose-5-phosphate; TCA, tricarboxylic acid; VHL, von Hippel–Lindau tumor suppressor.

and β2-adrenoceptors that make them responsive to noradrenaline and other signals that promote changes in their metabolic requirements[54]. Activation of the SNS leads to the rapid downregulation of *CXCL12* expression and the export of HSCs and other types of immune cells into the circulation. β2-adrenoceptors on MSCs can mediate changes in osteoblast differentiation and, ultimately, bone remodelling[54].

Biochemistry of the niche

The bone marrow niche has its own unique biochemistry, supported by a rich vasculature that makes it particularly suitable for stem cells. The endosteal surface of each trabecula is surrounded by bone marrow with constituent progenitor and stem cell populations. The endosteum also contains pre-osteoblastic cadherin-2-expressing cells, and has nerve and blood supplies[55], although these features are difficult to detect with plain-light microscopy. The heterogeneous environment of the endosteum contains scattered regions of microvascular infiltration, and other regions of relative hypoxia[56] that are highly attractive regions for HSC-homing[57–59]. Interestingly, quiescent HSCs tend to reside in areas of very low blood perfusion, whereas more proliferative HSCs with a lower reconstitution potential tend to prefer areas with greater blood flow[59]. The hypoxic microenvironment in the endosteum is a major factor in the integration of skeletal and haematopoietic functions, owing in part to the central role of hypoxia-inducible factor (HIF)-1α in HSC differentiation[60,61].

Metabolic reprogramming in hypoxia

Hypoxia induces a cellular response via a family of HIFs expressed in HSCs and MSCs that regulate a number of downstream signals. HIF transcription factors are composed of one of three oxygen-sensitive α-subunits — HIF-1α, HIF-2α or HIF-3α — and a constitutively expressed β-subunit, HIF-1β, also called aryl hydrocarbon receptor nuclear translocator (ARNT)[57]. Once the HIF-α subunit binds ARNT, the binary complex translocates to the nucleus and activates the transcription of genes containing hypoxia-responsive elements (HREs)[59]. In normal oxygen conditions, or when O_2 concentration exceeds 5%, HIF-1α protein is degraded by the proteasome within 5 min[62,63]. Three prolyl hydroxylase domain (PHD) enzymes hydroxylate two residues within the oxygen-degradation domain of HIF-1α, leading to ubiquitination and subsequent degradation of the HIF complex[62] (FIG. 3). Pharmacologic approaches to inhibit PHD proteins and stabilize HIF-1α are currently in clinical trials for the treatment of anaemia caused by chronic kidney disease and to enhance stem cell pools after chemotherapy and radiation therapy[58].

Survival and maintenance of HSC 'stemness' in hypoxia requires substantial metabolic adaptations. As noted above, relative hypoxia (in which O_2 concentration may be as high as 5%) induces the stabilization of HIF-1α in HSCs as well as the transcription of multiple downstream target genes including *VEGFA*[64]. Metabolic reprogramming of quiescent cells is necessary to prevent differentiation, and this reprogramming occurs through a shift from oxidative phosphorylation to glycolysis[65,66]. Importantly, glycolysis, although less efficient than mitochondrial oxidation in generating ATP, reduces oxidative stress and generation reactive oxygen species (ROS), both of which drive stem cell differentiation. Indeed, HSCs are particularly well-suited to tolerate oxidative stress through a well-organized antioxidant defence system.

MSCs also express HIF proteins and respond to hypoxia similarly to HSCs, with the upregulation of multiple HIF-inducible genes following translocation of the transcription factor complex to the nucleus[63]. Enhanced HIF-1α protein expression promotes osteogenic differentiation over adipogenesis in the marrow[67], potentially via increased production of VEGF-A, which is a potent angiogenic factor, and its subsequent suppression of peroxisome proliferator-activated receptor γ (PPAR-γ). VEGF-A has been shown to be essential for endochondral bone formation *in vivo,* and for MSC differentiation into osteoblasts through binding to VEGF receptor 2 (REF. 68). Other HIF-inducible proteins in MSCs include lactate dehydrogenase A (LDH-A), phosphoglycerate kinase and glucose transporter 1 (GLUT1), all of which are essential for the promotion of glycolysis over oxidative phosphorylation and, ultimately, for osteogenic differentiation[67,69].

Two key proteins, GLUT1 and LDH-A, are both upregulated during hypoxic conditions in HSCs and MSCs via activation of the transcription factor HIF-1α. As noted, mitochondrial respiration must be suppressed to enable glycolysis to predominate; this suppression occurs through the hyperactivation of AMP kinase (AMPK) and downregulation of several relevant mitochondrial genes, as well as the suppression of ROS production. Activation of AMPK by metformin, an anti-diabetes drug, enhances glycolysis, but also suppresses HSC differentiation and maintains the stemness of these cells[65,70]. By contrast, metformin has been shown to enhance MSC differentiation, also through AMPK, by upregulating the master osteogenic transcription factor RUNX2 (REF. 71). Studies from independent laboratories have confirmed that osteogenic differentiation is driven almost exclusively by glycolysis and that GLUT1 is essential to that process[72,73]. Moreover, PTH, which can enhance HSC egress and MSC differentiation, works by inducing glycolysis in differentiated osteoblasts, probably through upregulation of *GLUT1* and *GLUT3* expression[74]. Overall, it is clear that the bioenergetics of cells in the niche, driven by ATP demand, help determine how specific transcriptional factors induce the differentiation of stem and progenitor cells.

Effects of growth factors

Biochemical changes in both HSCs and MSCs also occur as a result of vascular and paracrine delivery of cytokines and chemokines. For example, HSC mobilization occurs because of key receptor–ligand interactions, particularly SDF1–CXCR4 (REF. 75). Other ligands and their receptors are also integrated within the bone marrow niche, including integrin α-4–VCAM1 (vascular cell adhesion protein 1), L-selectin (CD62L)–PSGL-1 (P-selectin glycoprotein ligand 1, or CD162) hyaluronic acid (HA)–CD44, and mast/stem cell growth factor receptor Kit (c-Kit, or CD117)–Kit ligand[76]. Ephrin type-B receptor 4 (EPHB4) is a tyrosine kinase receptor that has been shown to modulate HSC fate through its ligand ephrin-B2 (REF. 77). In sum, it is apparent that growth factors signal the egress of HSCs from the marrow and also probably encourage the use of alternative metabolic pathways to support differentiative function or, in some cases, to inexorably alter stem cell fate.

Consequences of niche disruption

The intricate balance between HSCs and MSCs is subject to disruption by many factors, including tumour-cell invasion, excessive ROS production, substrate insufficiency and a host of drugs used to treat autoimmune diseases that might unintentionally harm the niche. Pathologic changes in MSC differentiation can also alter the niche and lead to activation of tumorigenesis via the Notch signalling pathway. For example, in a 2014 study, Krevvata *et al.*[78] demonstrated that constitutive activation of β-catenin interacts with Foxo1 in osteoblasts and MSCs, which in turn enhances the expression of the Notch ligand protein jagged-1. In long-term repopulating HSCs, chronic exposure to jagged-1 results in leukaemic transformation[79].

Cancer-related disruption of the niche

Niche colonization by tumour cells

The unique properties of the bone marrow niche make it exceptionally conducive to colonization by tumour cells (FIG. 4): the niche can be considered as 'fertile soil' for malignant cell 'seeds'. Breast cancer, prostate cancer and multiple myeloma cells strongly prefer to metastasize and grow within the bone marrow rather than other anatomical locations, not only because of its characteristic properties described above, but also due to positive-feedback loops initiated by tumour cells within the niche. By causing osteolytic (bone-destructive, common in breast cancer and multiple myeloma) or osteoblastic (bone-forming, common in prostate cancer) lesions, cellular crosstalk is initiated that supports tumour growth and uncoupling of bone remodelling. As reviewed elsewhere, osteolytic cancers induce a forward-feedback loop termed the 'vicious cycle', in which bone-embedded growth factors, extracellular matrix proteins, and calcium are released as bone is resorbed, which then signal to tumour cells, accelerating their proliferation[80,81]. When receptor activator of NFκB (RANK) on pre-osteoclasts is stimulated by RANK ligand (RANKL), produced by osteoblasts and tumour cells, osteoclast number and activity are increased, which directly supports multiple myeloma cells[82]. Osteoclastogenesis then leads to bone degradation via resorption pits and proteinases, such as cathepsin K, which can also be produced by tumour cells[83]. RANKL itself has also been identified as a tumour chemokine[84], creating an intricate web of signalling interactions between osteoblasts, osteoclasts, and tumour cells.

As tumour cells take hold and begin to grow in the favourable conditions of the bone marrow, they modulate and usurp the marrow to support their own growth at the expense of normal bone homeostasis, leading to increased fractures, hypercalcaemia, spinal cord compression, immune cell dysfunction, pain, and, eventually, death[85]. The microenvironment not only is required for this process, but also changes in response to tumour growth. For example, MSCs from patients with multiple myeloma and myelodysplastic syndrome are abnormal

due to the effects of local tumour cells, and have inhibited osteogenesis and increased tumour-supportive functions, which are driven through numerous mechanisms[86–88]. Similarly, leukaemic myeloid cells also hijack the normal osteogenic process by stimulating MSCs to overproduce functionally altered osteoblast-lineage cells, which accumulate in the bone marrow cavity as inflammatory myelofibrotic cells[89]. These myeloproliferative neoplasia-associated osteoblasts, in turn, exhibit decreased expression of many HSC-retention factors and have a severely compromised ability to maintain normal HSCs, but are more efficient at harbouring leukaemia stem cells[89]. Targeting this pathological interplay between osteoprogenitor-lineage cells and tumour cells represents a key avenue to treat myeloproliferative disease or bone metastasis. Hence, the 'fertile soil' of the bone marrow is not only responsible for the successful growth of tumour cell 'seeds', but is also, by the same metaphor, fertilized further by the tumour cells themselves, as they create a more hospitable environment for further tumour colonization and expansion.

Facilitating tumour survival

The bone marrow also provides protection from anticancer therapies through cell adhesion-mediated drug resistance (CAM-DR). This resistance is, in part, due to the quiescent state induced in cells within the bone marrow niche, which allows for long-term survival of malignant cells. The ability of the bone marrow niche to revert cells to a stem-cell state can cause tumour cells that are no longer clonogenic to revert to a stem-like state after signalling from the bone marrow via myofibroblast-derived factors such as hepatocyte growth factor[90]. Thus, cancer cell stemness is not a fixed state, but can be instilled and nurtured by the niche. The niche also provides numerous growth factors that facilitate tumour quiescence or growth; precisely what dictates tumour cell fate (that is, apoptosis, dormancy, self-renewal, or proliferation) is still unclear[91]. Importantly, the plasticity of differentiation that is characteristic of putative cancer stem cells (CSCs), which can be driven by bone marrow interactions, suggests that eradicating CSCs would not stop tumour growth as more CSCs could be reinitiated from dedifferentiation of non-stem cells upon association with the niche[92]. Although evidence for this mechanism is clear with leukaemia-initiating stem cells[90], the presence of stem cells or tumour-initiating cells remains controversial in other cancers, such as multiple myeloma, and hence the role of the bone marrow is likely to be dependent on tumour type and clonal properties.

Many molecules, including matrix metalloproteinases, VEGFs, activin-A, and connective tissue growth factor, facilitate tumour survival in the bone marrow and can act as cell-nonautonomous factors[93,94]. Bone-derived factors such as the TGF-β family cytokines and insulin-like growth factors stimulate tumours to activate osteoblasts via molecules such as VEGF, platelet derived growth factor (PDGF), and endothelin-1 (REFS 94,95). TGF-β (through Smad pathway signalling), hypoxia (through HIF-1α), and extracellular calcium (through protein kinase B (PKB) and MAPK pathways)

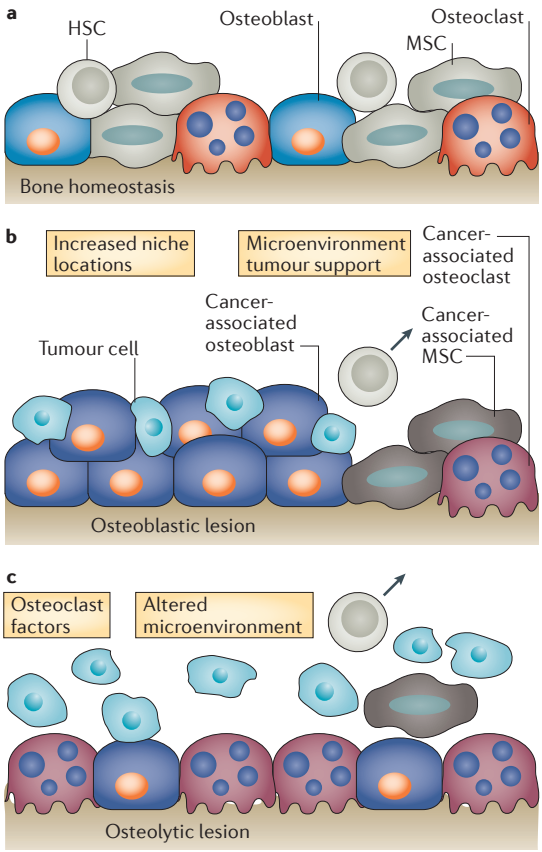

Figure 4 | **Cancer-related disruption of the bone marrow niche.** The niche is an attractive milieu for certain tumour cells, owing to a number of physical, biochemical and cellular properties. The relationship between the bone-marrow niche and infiltrating tumour cells is dynamic. **a** | Once tumour cells arrive in the bone marrow, they initiate a forward feedback mechanism to alter and hijack the niche, making the microenvironment even more hospitable for tumour cells. Depending on the tumour type, osteoblastic (increased bone) (**b**) or osteolytic (decreased bone) (**c**) lesions can occur, and feedback to accelerate tumour growth, drug resistance, and protection via dormancy of certain clones. HSC, haematopoietic stem cell; MSC, mesenchymal stromal cell.

alter gene expression within tumour cells in the bone microenvironment, enabling the survival and growth of these cells in the marrow cavity[94,96,97]. Many osteolytic tumour cells produce PTHrP, TGF-β, dickkopf-1, sclerostin, and RANKL; moreover, osteoblastic tumours often secrete bone morphogenic proteins and other growth factors (such as fibroblast growth factors and Wnt family members), but many of the mechanisms of osteoblastic lesion development remain undetermined[98]. Metabolic changes in tumour cells induced by their local microenvironment are also now recognized as important contributors to tumour growth and potential therapeutic targets[99].

Effects of bone marrow cells on tumours

The many cells that contribute to the 'fertile soil' of the niche have been reviewed previously and described

above[81,100,101]. Just as these cellular elements of the bone marrow are important in supporting and regulating the haematopoietic niche, they also have roles in the promotion, and perhaps the inhibition, of tumours within the bone marrow. For example, myeloid-lineage progenitor cells ($CD11b^+$), have been shown to support tumour growth, migration, and invasion *in vitro* and, when co-implanted with tumour cells, can promote metastasis *in vivo*[102]. Macrophages also are important in supporting multiple myeloma, other haematological malignancies, and metastatic tumours through contact-mediated and non-contact-mediated mechanisms[103], while the effect of bone marrow adiposity on tumour niche colonization is a new frontier in cancer research.

Osteoblasts are decidedly vital in maintaining HSC quiescence, but their putative effects on tumour cells are more controversial and include inducing dormancy, cell-cycle arrest, apoptosis, or proliferation, depending on the models, osteoblast cells, culture conditions and tumour cells used[88]. Osteoblasts might also have an anti-leukaemic role[104], and osteoblast numbers are significantly deceased in leukaemia[78]. Our review published in early 2015 discussed the roles of endosteal osteoblasts and bone marrow adipocytes in myeloma[88] and others have reviewed this topic for breast and prostate cancers[105]. The newly appreciated roles of osteocyte signalling, specifically the osteocyte-derived Wnt-inhibitor sclerostin, from lacunae to healthy and cancerous bone marrow niches, has also been reviewed in a 2014 publication[106]. Targeted reprogramming of interactions between bone marrow cells and tumour cells could prove to be a breakthrough in therapeutic approaches to inhibiting tumour growth in bone.

Within the bone marrow, MSCs have been shown to support tumorigenesis in a plethora of ways, for example by inducing angiogenesis and via secreted chemokine and contact-mediated paracrine signalling. MSCs can support growth, increased aggressiveness, and self-renewal of multiple myeloma, leukaemia, and other solid tumours *in vitro* and *in vivo* through the activation of pathways such as PKB, Ras, NFκB, HIF-1α, Myc, human telomerase reverse transcriptase and interferon regulatory factor[107], and by creating specific CSC niches through cytokine loops involving IL-6 and platelet basic protein (CXCL7)[108]. Myeloma-associated MSCs are distinct with regards to gene expression, function, proliferation and differentiation potential from MSCs from healthy donors, representing one example of how tumour cells manipulate their niche. This abnormal state of myeloma-associated MSCs seems to be induced by multiple myeloma cells[109,110], but it is possible that MSCs become abnormal as a pre-myeloma step that could lead to the initiation or propagation of myeloma.

Alterations in the malignant niche

Normal and malignant niches differ in many ways, depending on the type of tumour colonizing the bone and the extent to which the niche has been altered. In general, the accumulation of malignant cells in the bone marrow interferes with feedback signals for normal haematopoiesis, which results in cytopenia[111]. In osteolytic niches, which are common with breast cancer and almost exclusively found with multiple myeloma niche colonization, bone formation and resorption activities are uncoupled such that osteoclast activity is increased and osteoblast activity inhibited. In osteoblastic malignant niches, which are common in prostate cancer metastasis, the niche is skewed towards increased numbers and activity of osteoblasts and decreased osteoclast activity. As discussed earlier in this article, tumour-associated MSCs are abnormal[109] and often the immune system is dysregulated, with increased numbers of regulatory T cells and other immune-inhibiting cells, and decreased activity or numbers of effector T cells and other cells that have the capacity to kill tumour cells[112]. Tumour-associated vasculature is often faulty, not only enabling the formation of vessels to feed growing tumours, but also creating leaky neovasculature, making it more difficult to deliver drugs to tumours, many of which become hypoxic and necrotic in the centre once they have grown large enough[113]. Messenger RNA and microRNA signatures, as well as exosome content and volume from bone marrow stroma, were also found to be abnormal in samples from patients with multiple myeloma[110,114]. Notch receptor signalling[115] (a critical regulator of HSC fate and differentiation in the bone marrow) and the SDF1–CXCR4 axis are often deviant[116], extracellular matrix components and ratios are often abnormal[117], and changes in glycosylation of cell-surface adhesion molecules, such as selectin ligands, integrins and mucins[118], in cells of the bone marrow are often observed in malignant niches.

Haematopoietic stem cells and tumour cells

Parallels in niche homing and colonization

Although the 'vicious cycle' propagates through destructive mechanisms that are absent from healthy HSC–bone marrow interactions, the initial stages of homing to the bone marrow progress similarly for HSCs and tumour cells (FIG. 5). Many of the same pathways, proteins, and adhesion molecules involved in HSC trafficking to the bone marrow niche are used by metastatic tumour cells, and both cell types show a preference for the highly vascularized metaphysis of the bone[119]. Rather than central marrow, the endosteum seems to be the preferential site for HSCs and tumour cells[120]. Ligand–receptor interactions of adhesion molecules, such as SDF1–CXCR4 (REF. 121), integrin α-4–VCAM-1, CD44–osteopontin, and integrin $\alpha_v\beta_3$–bone sialoprotein[122], are important in the homing of HSCs and tumour cells to bone[123]. Calcium receptors also have a role in the niche-homing of HSCs and in the homing and proliferation of cancer cells[123]. PSGL-1 is expressed at high levels in multiple myeloma cells and its interaction with selectins in the bone marrow enhances adhesion and homing of these cells. Altered glycosylation and expression of sialyltransferases in multiple myeloma cells also affects their adhesion and migration, specifically into the bone marrow[117,124].

Bone metastasis-specific patterns of messenger RNA and microRNA expression within cancer cells

often contain similarities to those of HSCs, providing additional insight into how tumour cells mimic HSCs in homing to bone marrow[85,97,125]. On a larger scale, physical features of the bone marrow microenvironment, including acidic pH, high extracellular calcium concentrations, and adjacent sinusoidal blood vessels, also enhance bone colonization by tumour cells (as they do for HSCs) and contribute to the vicious cycle, as described previously[97,126]. Hypoxia has been implicated both in the induction of tumour quiescence[80] and in egress of tumour cells from the marrow[127]; these effects, often driven by HIF signalling, are also seen in HSCs[127–129]. As described above, hypoxia is a characteristic of the bone marrow, but whether the HSC niche (or tumour-homing niche) is always or necessarily hypoxic is still contentious, as HSCs and tumour cells are often found in close proximity to blood vessels[130]. Nonetheless, hypoxia, via HIF-1α, can induce quiescence in HSCs and tumour cells by suppressing mitochondrial oxidative metabolism and promoting anaerobic glycolysis, which is thought to be the main source of ATP in HSCs[130]. HIF-1α has also been shown to be constitutively expressed by multiple myeloma cells in 35% of patients, independent of actual hypoxic conditions; furthermore, an antisense oligonucleotide inhibiting HIF-1α induced permanent cell-cycle arrest in multiple myeloma cells in preclinical studies[131], suggesting that targeting HIF-1α in multiple myeloma cells, in either hypoxic or nonhypoxic environments, could hold great clinical promise. The bone marrow microenvironment also induces quiescence and drug resistance in tumour cells in the bone marrow, owing in part to metabolic changes such as increased expression of HIF-1α and LDH-A[132].

Competition for the niche

Numerous studies have demonstrated that multiple myeloma or bone-metastatic tumour cells compete with HSCs for the niche, inhibiting haematopoiesis by displacing HSCs[101] (FIG. 5). Bone-metastatic prostate cancer cells have been described as 'parasitizing' the bone marrow niche; via annexin A2 and its corresponding receptor, these cells bind to osteoblasts and become quiescent[133]. Binding of prostate cancer cells to osteoblasts can also induce expression of TANK binding kinase 1 (TBK1), which leads to drug resistance via inhibition of serine/threonine protein kinase mTOR[134]. Prostate cancer cells seem to displace resident HSCs from the niche[135], and patients with multiple myeloma have been found to have decreased numbers of haematopoietic progenitor cells[136]. These and other studies demonstrate that tumour infiltration inhibits marrow haematopoiesis and can cause anaemia, leukopenia and bone marrow failure by competing with mature haematopoietic cells and HSCs for the same niche[137].

As prostate cancer is one of the only bone-metastatic tumours to cause osteoblastic bone disease, the interactions between prostate cancer and the niche are probably very different from interactions between osteolytic tumours (multiple myeloma and breast cancer) and the niche. The effects of osteoblasts within the HSC niche on the quiescence and drug resistance of disseminated prostate cancer cells suggests that the induction of more osteoblasts would be advantageous to these tumours. Breast cancer and myeloma cells that hijack the HSC niche would presumably gain the same advantages, yet breast cancer and multiple myeloma cells do not increase osteoblast numbers but rather inhibit osteoblast differentiation; this difference suggests that osteolytic tumours benefit more from tipping the balance towards osteoclastic activity than from inducing more bone marrow niches[138]. The reasons for the development of osteolytic versus osteoblastic lesions, and the differential effects of these lesions on the niche, requires further investigation.

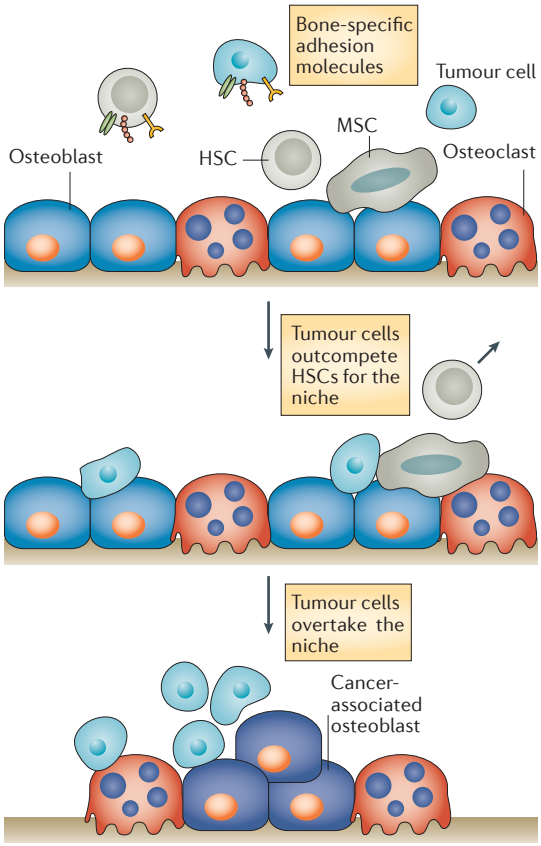

Figure 5 | **HSCs and tumour cells compete for the bone marrow niche.** By mimicking haematopoietic stem cells (HSCs), bone marrow-homing tumour cells often use the same signalling pathways as HSCs to colonize the niche. Eventually, tumour cells outcompete HSCs for the niche, physically displacing present cells and monopolizing the niche to block further homing of HSCs. This leads to disrupted haematopoiesis and subsequent dysregulation of the niche itself. MSC, mesenchymal stromal cell.

Pre-metastatic niches

Bone loss in malignant or nonmalignant disease is caused by an imbalance between bone formation and bone resorption; increased osteoclast resorption and/or reduced osteoblast bone formation causes pain, hypercalcaemia, fracture, and disrupted haematopoiesis[138]. The bone marrow niche becomes dysfunctional in

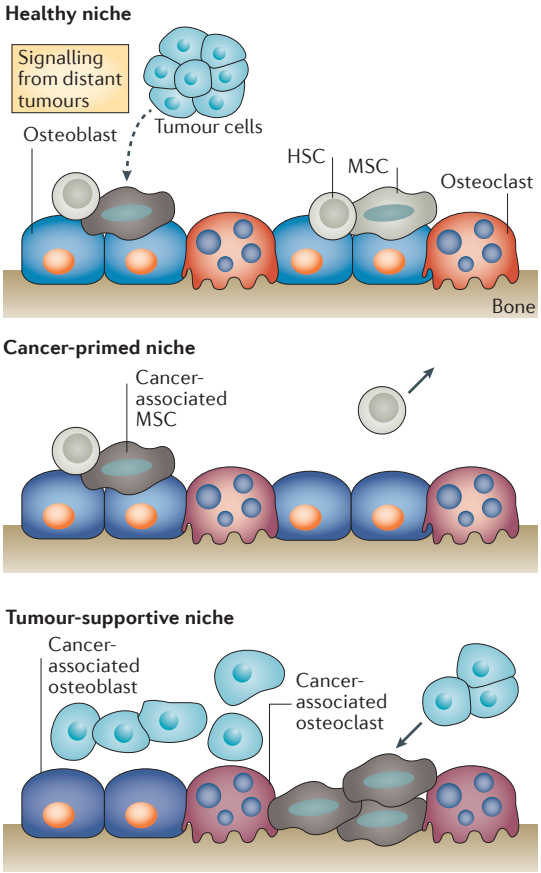

Figure 6 | **The pre-metastatic niche.** Alterations in the bone marrow niche can affect the host response to malignancies. Pre-metastatic niches can be initiated in the bone marrow by distant tumour cells that send signals (e.g. within exosomes) to precondition the niche, facilitating subsequent tumour-cell infiltration and colonization of the niche. HSC, haematopoietic stem cell; MSC, mesenchymal stromal cell.

malignant and nonmalignant bone diseases, and niche dysfunction is also a potential initiator of these diseases — a dilemma that often complicates aetiology and determination of disease origin. Several studies have shown that changing the bone microenvironment before the introduction of cancer cells, using either bortezomib (a bone anabolic agent) in multiple myeloma[139] or breast cancer models[140] or anti-SDF1 pretreatment in a multiple myeloma model[121], can make it a less-hospitable environment for tumours.

The concept of a pre-metastatic niche, or an area that is altered to create an environment specific for metastasis before tumour cells physically arrive, has some provocative support, both in the bone marrow and in other regions[141] (FIG. 6). By sending out signals to the bone marrow, tumour cells may precondition the niche to facilitate its later colonization. Evidence of this phenomenon has come from a 2015 breast cancer study showing that tumour-secreted protein-lysine 6-oxidase (lysyl oxidase) can cause the formation of pre-metastatic niches in the distant bone marrow by modulating osteoclasts and osteoblasts, and by stimulating osteolysis (hence initiating the vicious cycle), before tumour cells arrive[142]. In another breast cancer study, peripheral blood serum from patients with cancer significantly supported tumour growth and trans-endothelial migration, compared with serum from healthy donors, and contained higher concentrations of PDGF-AB, intercellular adhesion molecule 1 and vascular cell adhesion protein 1, which could aid tumour extravasation, bone resorption and proliferation[143]. Signals from tumour cells can be transmitted in exosomes or other microvesicles or by circulating free DNA to create distant pre-metastatic niches[144–146]. MSCs have also been found to be recruited to regions of pre-metastatic niches and, through prostaglandin E2 induced by primary tumour-derived VEGF, create a region more susceptible to tumour colonization[147]. Kerr *et al.*[148] have shown that platelets are pivotal for tumour communication with the bone marrow when creating a pre-metastatic niche, whereas others have found bone-marrow-derived myeloid cells to be crucial in creating pre-metastatic niches in lung[141,149]. In summary, it seems that tumours probably induce systemic changes and emit systemic signals to hijack, remodel and support tumour-cell colonization of the bone marrow niche[150].

Niche-directed carcinogenesis

Not only does the bone marrow niche support metastatic tumour cells arriving from distant locations, but it might also have a role in the first tumorigenic event of oncogenesis (FIG. 7). A 2010 study by Raaijmakers *et al.*[151] demonstrated that alterations to the normal HSC niche (via deletion of *Dicer1* specifically in mouse osteoprogenitors, but not in mature osteoblasts) disrupt the integrity of haematopoiesis and induce myelodysplasia and secondary leukaemia. A subsequent review article by Raaijmakers summarizes much of the work examining how modulations in ancillary cells of the bone marrow could directly cause leukaemogenesis, myelodysplasia and myeloproliferative disorders through ROS formation (inducing DNA damage), by affecting cell-cycle arrest, or via other pathways[90]. These data call into question the dogma that cell-autonomous events lead to initiation of cancer, by suggesting that leukaemias might develop through non-cell autonomous pathways, which might also be true for other haematological malignancies such as multiple myeloma. In summary, the niche might also act as a tumour-enabling milieu by priming or directly initiating tumorigenesis; this concept deserves further investigation.

Recreating the niche experimentally

Three-dimensional *in vitro* models are vital to the correct recapitulation of bone and cancer interactions, as they more accurately capture the physiological interactions and cell–cell signalling within the bone marrow niche than 2D models. Some researchers have used matrigel[152] and other hydrogels as 3D substrates to explore the tumour-supportive effects of MSCs and other properties of the niche, or have expanded 2D cultures on cellulose membranes using perfusion bioreactors to create multi-cell layered, although not

porous or trabecular-like, bone mimics[153]. However, to capture the realistic mechanical properties of the trabecular environment in which tumour cells grow in the bone marrow, we and others have utilized harder, mineralizable biomaterials with pores similar to those of trabeculae, such as silk scaffolds[110], polycaprolactone-tricalcium phosphate (PCL-TCP) scaffolds[154], or calcium phosphate scaffolds[155], with or without bioreactors or spinner-flasks[156,157]. The development and use of bioreactors or spinner-flasks for bone tissue engineering, led by the laboratories of Langer, Kaplan and Vunjak-Novakovic among others, often facilitates better nutrient diffusion of biopsies or tissue-engineered samples, allowing for the development of longer-term models with better osteogenic differentiation and mineralization versus soft, hydrogel models, although with potentially increased challenges for imaging or tumour-cell growth[156–159].

The vascular niche within the bone marrow is another important area that researchers must recapitulate in the bone marrow *in vitro*[113]. In our models, this was done using RFP-labelled human umbilical vein endothelial cells in co-culture with GFP-labelled MM.1S myeloma cells[110]. Other models include 3D spheroid tri-cultures of osteoblasts, endothelial cells, and prostate cancer cells in microfluidic devices[160]. Still others have used silk tubes, microfluidic devices, megakaryocyte culture systems, and other types of 3D tissue engineering techniques to model the bone marrow vasculature[161–163].

Many models also exist to study bone marrow metastasis *in vivo*. For these models, the researcher must decide what to use in terms of the tumour cell line (or primary cells), the model for bone metastasis or growth in the bone marrow, the mouse model and/or strain, and the methods to track tumour burden and bone parameters. Examples of commonly used models of metastasis to the host bone morrow are intravenous injection of the myeloma cell line MM.1S[139,164], intracardiac injection of the breast cancer cell line MDA-MB-231 (REFS 165,166), and intracardiac injection of the prostate cancer cell line PC-3 (REF. 167). An advantage of these models is that they recapitulate the latter stages of bone marrow metastasis, including circulation of tumour cells through the bloodstream, rolling and arrest at a distant bone marrow capillary, extravasation across the endothelial lining, colonization of the new microenvironment, proliferation, and hijacking and reprogramming of the bone marrow. As most of these steps are considered rate-limiting, with only a fraction of tumour cells making it through each step, these are typically seen as excellent models for bone metastasis. However, these models do not recapitulate the early stages of bone marrow metastasis, specifically the epithelial-to-mesenchymal cell transition, escape through the basement membrane, and intravasation (the stage where the cancer cell first enters into circulation), which are all necessary steps of distant metastasis for any solid primary tumour.

Another advantage of the injection models described is that they use human, rather than mouse,

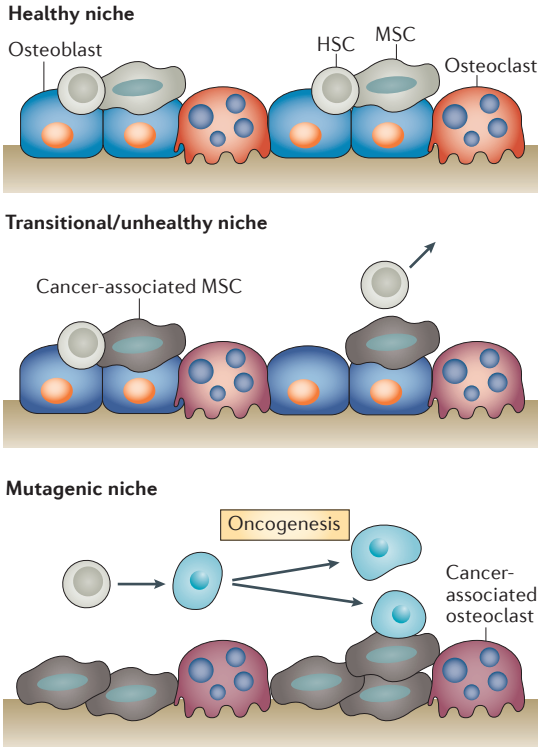

Figure 7 | **Niche-directed carcinogenesis.** Alterations in the bone marrow niche can directly initiate malignant transformation. Niche-directed carcinogenesis is a phenomenon (demonstrated in mice) whereby the niche itself becomes abnormal, which then causes *de novo* tumorigenesis. Validating this phenomenon in humans will be crucial to understanding the physiological roles of the niche in tumour initiation. HSC, haematopoietic stem cell; MSC, mesenchymal stromal cell.

tumour cells. However, xenograft models require the use of immunocompromised mice, such as Nod/SCID gamma (NSG) or *Rag2*$^{-/-}$ mice, and thus cannot accurately capture the roles of the immune system in cancer. Therefore, certain transgenic or syngeneic models (where tumour cells from transgenic mice are directly injected into mice from the same background, to create models of accelerated tumour growth), such as the 5TGM and Vk*Myc multiple myeloma models, are often used to preserve the immune system component of the disease[168,169].

Importantly, tumour cells often do not colonize the bone marrow after circulatory injection, for interrelated reasons such as tumour clonality, heterogeneity, lack of the traits necessary for bone-homing and engraftment, and entrapment in the lungs, spleen, or other organs. To get tumour cells to grow within the bone marrow, researchers often must use direct orthotopic injections, omitting the steps of extravasation and bone-homing. For example, Medyouf *et al.*[86] have developed a primary patient co-transplantation model using a xenotransplant (intrafemoral injection) of tumour cells in sublethally irradiated NSG mice. This elegant work demonstrates the ability of myelodysplastic syndrome disease cells to reprogramme progenitors in the bone marrow

microenvironment, and illustrates how MSCs facilitate myelodysplastic-cell engraftment — two recurrent themes in the role of MSCs and cancer. Models of direct tumour cell injection into mouse bones (intratibial or intrafemoral[86,170]) are advantageous in that they contain real, complete, vascularized, functional bone marrow niches; however, the aggressive injection of a bolus of tumour cells into this niche causes inflammation, bone destruction, and formation of a non-vascularized tumour that has not grown or progressed in response to its bone marrow environment (at least initially). Still, after healing, tumour growth within the bone marrow can be studied and these models have proven important and effective for studying bone–tumour interactions, understanding bone marrow niche destruction, and developing therapeutics[166].

Newer models of bone marrow metastasis use subcutaneously implanted bones, which, depending on the system, allow for several features: easier optical imaging; a more-controlled environment; the existence of multiple identical bone marrow niches within the same mouse; human bone extracellular matrix and cellular constituents; and a potentially less-inflammatory bone marrow niche (versus direct injection of tumour cells into bone). These models variously use implanted human bone (fetal bone or adult bone from hip replacement surgery; known as a SCID-hu model)[171], rabbit bone (SCID-rab model)[172], or mouse bone (SCID-mu model)[121]. Tissue-engineered bone (TE-bone) models from silk scaffolds[114,173–175], calcium phosphate scaffolds[155], or other biomaterials are also now being utilized as more controllable, reproducible bone-marrow-niche models to investigate the contributions of bone marrow stroma to tumour engraftment or metastasis. For example, one silk-scaffold model utilizes bone marrow stromal cells that are seeded onto silk scaffolds and differentiated into TE-bone that can then be used to study breast cancer metastasis[175]. In this way, different cellular or biochemical components of the bone-marrow microenvironment can be specifically studied for their individual contributions to bone-marrow metastasis. Effects on tumour growth can also be studied by directly injecting tumour cells into TE-bone after implantation or by co-seeding tumour cells into TE-bone cultures before implantation. Paton-Hough *et al.*[166] have comprehensively reviewed current *in vivo* myeloma models, and the drawbacks, advantages, and caveats of each.

Challenges remain in fully recapitulating the niche *in vivo* and *in vitro,* in part because of the dynamic nature of the microenvironment. As noted, hypoxia is a critical element of the niche, not only for maintaining stemness, but also for inducing glycolytic pathways that maintain the vitality of the niche. Hence, the bioenergetic pathways of the niche must be faithfully maintained, which requires specific nutrient and oxygen concentrations in the media. Similarly the cell–cell interactions described above, whether related to malignant-cell communication with stromal elements or MSC–HSC exchanges, have unique requirements and stages of differentiation that are difficult to restore completely in an artificial environment. Notwithstanding these obstacles, accurate redefinition of the niche *in vitro* will provide significant insights and important platforms for testing new therapies.

Translational implications

Disorders of the bone marrow niche are manifest in several disorders, including myelodysplastic syndromes, myeloproliferative disorders, aplastic anaemias, leukaemias, metastatic diseases, polycythemia vera, and thrombocytosis. Skeletal disorders, including some primary and secondary osteoporotic syndromes, can be considered disorders of the niche. Postmenopausal osteoporosis can be heterogeneous in terms of its aetiology relative to the bone marrow niche. In some women, the underlying disorder is an impairment in bone formation, which may be attributable to defective MSC recruitment. On the other hand, some women have increased bone resorption which is related to higher RANKL production and may be due to a combination of greater number of $CD4^+$ T cells and/or a suppression of regulatory T cells[176]. Thus, drugs for treating osteoporosis that target osteoblasts or their progenitors could have substantial effects on HSCs. The most frequently used 'anabolic' agent is PTH(1–34), which enhances osteoblast differentiation and builds bone mass[177]. PTH also induces HSC differentiation and can enhance mature peripheral erythroid and myeloid elements[24]. On the other hand, agents such as bortezomib, a proteasome inhibitor used to treat myeloma, may also induce MSC differentiation into mature osteoblasts, increasing bone mass and reducing myeloma progression in the marrow[139,178]. As the niche becomes more completely characterized *in vivo* and recapitulated *in vitro*, better therapies for chronic haematologic disorders, malignancies, and skeletal diseases will undoubtedly emerge.

One offshoot of work to define the bone marrow niche is the therapeutic use of MSCs. Although ~400 trials have been conducted using MSCs to treat a wide range of disorders, neither the FDA nor the European Medicines Agency has yet approved any MSC therapy[2]. Thus, over the past two decades, despite a plethora of publications and the promise for clinical applications, the role of MSCs in the treatment of heart disease, Alzheimer disease, diabetes mellitus or osteoporosis remains to be determined.

Conclusions

In summary, the bone marrow niche provides a home for HSCs and MSCs. The niche supports the integration of two major organ systems, the skeleton and the marrow. Even subtle alterations in the niche, whether biochemical or cytological, can lead to chronic diseases and could affect the host response to, or directly initiate, malignancies. Approaches using MSCs to treat common disorders are still experimental yet are relevant to the design of therapeutics that target the niche. As such, a more-complete understanding of the biology of this unique microenvironment within the bone marrow must, without doubt, continue to be a major research priority.

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Review

Dynamic interplay between bone and multiple myeloma: Emerging roles of the osteoblast

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ABSTRACT

Multiple myeloma is a B-cell malignancy characterized by the unrelenting proliferation of plasma cells. Multiple myeloma causes osteolytic lesions and fractures that do not heal due to decreased osteoblastic and increased osteoclastic activity. However, the exact relationship between osteoblasts and myeloma cells remains elusive. Understanding the interactions between these dynamic bone-forming cells and myeloma cells is crucial to understanding how osteolytic lesions form and persist and how tumors grow within the bone marrow. This review provides a comprehensive overview of basic and translational research focused on the role of osteoblasts in multiple myeloma progression and their relationship to osteolytic lesions. Importantly, current challenges for *in vitro* studies exploring direct osteoblastic effects on myeloma cells, and gaps in understanding the role of the osteoblast in myeloma progression are delineated. Finally, successes and challenges in myeloma treatment with osteoanabolic therapy (i.e., any treatment that induces increased osteoblastic number or activity) are enumerated. Our goal is to illuminate novel mechanisms by which osteoblasts may contribute to multiple myeloma disease progression and osteolysis to better direct research efforts. Ultimately, we hope this may provide a roadmap for new approaches to the pathogenesis and treatment of multiple myeloma with a particular focus on the osteoblast.

Contents

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Introduction

Multiple myeloma is an incurable plasma cell dyscrasia, a type of B-cell cancer that progresses through stages from monoclonal gammopathy of undetermined significance (MGUS), to asymptomatic smoldering myeloma, and lastly, to overt, symptomatic myeloma. This last stage is associated with significant morbidity, particularly in the form of fractures. Recent reports show that MGUS is also correlated with enhanced skeletal risks and osteopenia at this early stage of plasma cell transformation [1]. During multiple myeloma progression, osteolytic lesions are found throughout the skeleton with multiple tumors or "omas" packing the bone marrow. Osteolysis, a hallmark of multiple myeloma-induced bone disease, results from decreased osteoblastic activity and increased osteoclastic activity, releasing growth factors and cytokines embedded in bone matrix to form a "vicious cycle" [2–5]. The degree of osteolysis is an important parameter in the assessment of multiple myeloma patients. While the numbers of osteoblasts and bone formation rates are often increased in the early stages of tumor burden, due to increased osteoclast activity (which feeds back to activate increased osteoblast activity), these numbers become significantly lower when plasma cell infiltration occupies more than 50% of bone marrow [6]. Although bone-building (bone anabolic) treatments are currently being explored in early clinical trials to delay the time to first skeletal-related events (SREs) [7,8], much work remains to be done to validate if these are truly anti-myeloma strategies with long-term clinical benefits.

Preliminary research has demonstrated that osteoblast numbers can be decreased in hematologic malignancies, even in non-osteolytic tumors (a decrease of 55% was found in myelodysplasia and acute myeloid leukemia patients) and that osteoblasts can have an anti-tumor effect in blood cancers [9]. In support of this concept, the genetic depletion of osteoblasts in mouse models of acute leukemia led to increased circulating tumor cells and tumor marrow and spleen engraftment, higher tumor burden, and shorter survival [9]. Myelopoiesis increased and was coupled with a reduction in B-lymphopoiesis and compromised erythropoiesis, suggesting alterations in hematopoietic differentiation. When mice with acute myeloid or lymphoblastic leukemia were treated with a pharmacological inhibitor of duodenal serotonin, a hormone that suppresses osteoblast numbers, osteoblast numbers were increased, as expected. Remarkably, this treatment and subsequent maintenance of the osteoblast pool restored normal marrow function, reduced tumor burden and prolonged survival [9]. Therefore, osteoblasts may play a fundamental role in propagating leukemia in the marrow; pathways mediating this regulation still need identification.

One of the most pressing gaps in multiple myeloma biology is a basic biological understanding of the role of osteoblasts in disease progression (see Fig. 1). Recently, bone microstructural changes have been identified, along with elevated DKK1 and MIP-1α levels, in patients with MGUS [10]. Moreover, epidemiological data have demonstrated that low bone mineral density, increased fracture rate, and osteoporosis correlate with MGUS [1]. This provides more evidence that decreased osteoblast number/function, or weaker bones, could not only result from, but also cause or accelerate multiple myeloma [11]. As reviewed here, *in vitro* and *in vivo* studies to interrogate this hypothesis are crucial to elevate these correlations to mechanistically defined causal relationships. Studies are ongoing to identify underlying biological mechanisms by which osteoporosis could contribute to the development of multiple myeloma, and to gain insights into the roles of bone strength and bone-matrix forming cells in the etiology and pathogenesis of the disease. These studies are focused on several key questions: Do osteoblasts typically inhibit or stimulate the growth of myeloma cells? Would augmenting this specific cell type within the microenvironment decelerate or accelerate the progression of the disease, or affect its initial establishment? In which ways do osteoblasts directly or indirectly, through interactions with other bone marrow cells, affect the pathogenesis of multiple myeloma? Herein we review current concepts that begin to address these questions.

Ontogeny and developmental biology of the osteoblast

Osteoblasts are highly specific bone cells lining and formulating the mineralized matrix of the skeleton. They result from the osteogenic differentiation of mesenchymal stem cells (MSCs) and pass through a series of pre-osteoblastic stages as osteoprogenitor cells [12], until they become fully functional osteoblasts. When making bone, osteoblasts first deposit a dense organic extracellular matrix, primarily collagen I, and then harden this matrix by producing an inorganic calcium and phosphate-based mineral, hydroxyapatite. Different types of bone are formed by osteoblasts throughout the skeleton during skeletogenesis, remodeling, and fracture healing, including lamellar bone and woven bone [13]. During embryonic development, bone forms predominantly through a complex process termed endochondral ossification, a process including an intermediate cartilage stage [14]. A smaller fraction of human bones, such as the plates of the skull, are formed by intramembranous ossification, a process of direct differentiation of MSCs into mineralizing osteoblasts.

Osteoblasts in distinct anatomical locations respond uniquely to different stimuli and would likely respond differently to tumor cells, complicating studies aimed at using osteoblasts to inhibit multiple myeloma and other osteolytic cancers. What governs osteoblast phenotype and bone turnover in different bone compartments is largely unknown, but much work has been done to unravel the signaling mechanisms, pathways and relationships governing osteogenesis [15,16]. In 2009, Colnot [17] provided direct evidence that the major sources for skeletal stem cells are the periosteum, endosteum, and bone marrow and that while each give rise to osteoblasts, only the periosteum gives rise to chondrocytes, implicating different cellular populations within each distinct microenvironment. The periosteum also contributes to the growth and healing of long bones, demonstrating important differences in cell populations within various anatomical locations [18]. Recent evidence demonstrates that Wnt16 knockout mice have lower cortical bone mass, but no changes to their trabecular bone mass [19], whereas prior reports provide evidence that Wnt10a is necessary for trabecular bone formation, but not for cortical bone formation or maintenance [20,21]. These studies, and others using *Klotho*, *Src*, *and Sfrp4* null mice [22], demonstrate that osteoblasts and osteoclasts from different anatomical locations respond differently to ligands, trauma/disease, and treatments. This is also found clinically, where some therapeutics show different effects on long bones compared to vertebrae, or cortex versus trabeculae [23]. In sum, these studies suggest that osteoblast progenitors derived from these different locations may have disparate effects on bone remodeling and possibly cancer growth. This is a key nuance often ignored but which must be thoroughly understood before effective bone anabolic agents can be designed and targeted successfully.

Effects of osteoblasts on multiple myeloma

Unlike bone marrow MSCs, which support myeloma disease progression [24–26], evidence suggests that osteoblasts may suppress myeloma [27]. Osteoblast-derived growth factors play a large role in stimulating the growth of prostate cancers within the bone [28], raising the question of why this does not occur in myeloma. It is not clear if myeloma cells respond differently to these same osteoblast-derived factors, or if myeloma cells, like prostate cancer cells, actually benefit from osteoblasts, but proliferate even more strongly when they activate osteoclastic activity rather than osteoblastic activity. It is interesting that, although very rare, myeloma can also cause osteosclerostic lesions, without other symptoms of POEMS syndrome, suggesting again the possibility that osteoblastic activity may not necessarily be detrimental to plasma-cell-proliferative disorders [29–33].

As recently reviewed by Olechnowicz and Edwards [34], there are numerous other components of the host bone marrow that contribute to the pathogenesis of multiple myeloma, including fibroblasts, immune cells, adipocytes, endothelial cells, and osteoclasts. Contributions from

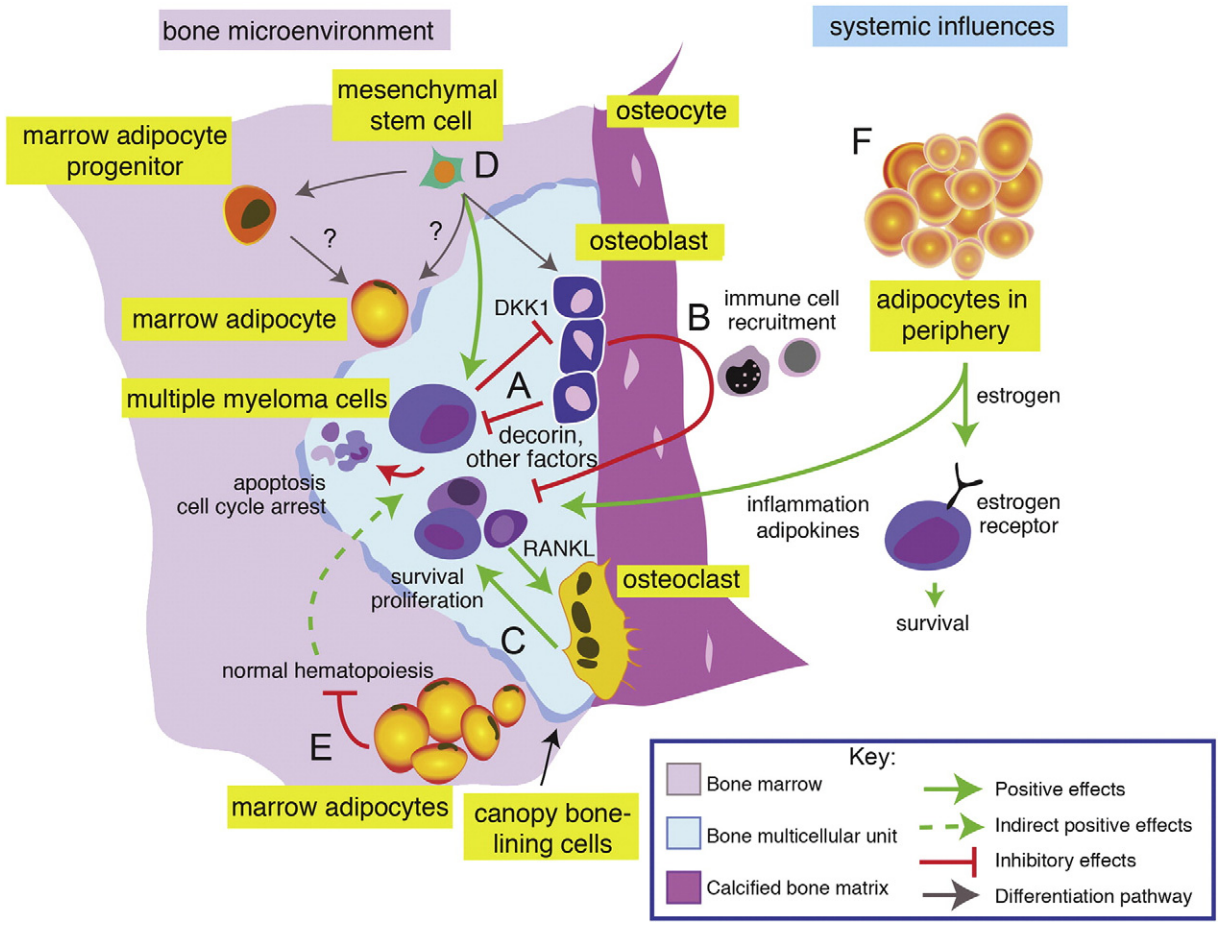

Fig. 1. The osteoblast as a central mediator of multiple myeloma growth. Multiple myeloma is a disease of the plasma cell. Multiple myeloma tumor cells grow within the bone microenvironment. Increasing evidence shows that osteoblasts play a central role in regulating the growth of multiple myeloma in the bone marrow through direct interactions or influences on other bone marrow niche cells. Within the bone microenvironment (left), osteoblasts secrete factors such as decorin (A) that directly lead to myeloma cell apoptosis and cell cycle arrest. In a reciprocal interaction, myeloma cells suppress osteoblast generation via DKK1. In addition, osteoblasts recruit immune cells to the bone marrow (B) where they can have anti-tumor effects, although recruitment of regulatory T cells and myeloid-derived suppressor cells can promote myeloma growth by inhibiting the anti-tumor immune response. (C) Increased osteoblastic activity leads to increased osteoclast activity, which can promote the survival and proliferation of myeloma cells. In turn, myeloma cells increase osteoclastic activity. (D) The mesenchymal stem cell within the marrow niche can have direct positive effects on myeloma cells and also determines the balance of resident osteoblasts and adipocytes. (E) The contribution of marrow adipocytes is still under active investigation, but marrow adipocytes may suppress normal hematopoiesis, leading to the development of myeloma cells. Other systemic influences (right) include adipose tissue, which, under conditions of excess adipocyte accumulation, induces systemic inflammation and release of adipokines and estrogen that may promote myeloma growth and survival.

the sympathetic nervous system and abnormalities in the myeloma-associated extracellular matrix also can support multiple myeloma progression. Perturbation of the osteoblast can lead directly, and spontaneously, to myelodysplasia or AML [35,36], demonstrating the critical influence of the bone microenvironment on hematological malignancies.

Direct effects of osteoblasts on myeloma growth

Biology of direct effects of osteoblasts on myeloma cells

Osteoblasts have been reported to directly inhibit multiple myeloma cells *in vitro*. One group demonstrated that some osteoblastic cells (MC3T3-E1 pre-osteoblastic cells and bone marrow-derived stromal cells), when differentiated into mineralized osteoblasts, induce apoptosis and cell cycle arrest in myeloma cells (i.e., cells such as RPMI8226, U266, KMS-12, INA6, 5TGM1, and primary patient samples) [27]. Decorin, the main small leucine-rich proteoglycan produced by osteoblasts, has also been identified as an endogenous, osteoblast-derived factor that suppresses multiple myeloma cell growth and survival [37]. In general, however, there is controversy about the net effect of osteoblasts on myeloma cells, as osteoblasts also produce factors that could support myeloma growth, such as osteocalcin, osteopontin, fibroblast growth factors, and transforming growth factor beta family members, although direct studies on this are lacking. One study demonstrated that quiescent myeloma cells prefer to reside in the endosteal/osteoblastic regions of the bone marrow compared with the vascular regions or spleen, indicating that osteoblasts may play a unique role in maintaining myeloma cells within a specific niche [38]. Another study showed that osteoblasts may be either supportive or inhibitory of multiple myeloma cells, and interestingly, these effects were dependent on the patient source of myeloma cells [39,40]. A better understanding of the direct anti-myeloma effects of osteoblasts is mandatory before bone anabolic treatments can be used successfully to inhibit multiple myeloma progression.

In vitro challenges of studying osteoblasts and myeloma interaction

There are several challenges that must be overcome to understand the role of osteoblasts in impeding myeloma growth. First, *in vitro* co-culture studies with plasma cells and osteoblasts are limited by the lack of relevant osteoblast cell lines. The human bone marrow stroma cell lines HS-5 and HS-27 do not differentiate into osteoblasts, and other cell lines that do mineralize, such as Saos2 [41] and MG-63 [42], are actually osteosarcoma rather than osteoblast cell lines. Certain cell lines, such as the human fetal osteoblastic cell line hFOB1.19 [43], which proliferate at 33.4 °C and differentiate at 39.4 °C or in osteogenic

medium, have been explored in multiple myeloma *in vitro* cultures [44] and could be exploited further. The two best options for osteoblast models may be primary human osteoblasts [45], or primary bone marrow-derived MSCs, which can be expanded and then induced to differentiate into mineralizing osteoblasts [24,46]. The challenge with using differentiated MSCs to model osteoblasts is delineating the moment when an MSC becomes a "pre-osteoblast," or has matured into a fully differentiated osteoblast, or has overshot the osteoblast stage to become an osteocyte. If the MSC is not differentiated far enough down the osteogenic pathway, it may still appear as a supportive stromal cell, accelerating the growth of myeloma cells rather than inhibiting them, as it is believed that osteoblasts may do *in vivo*. The mouse cell line MC3T3-E1 [47] is a well-accepted albeit unique pre-osteoblast cell line that undergoes linear osteogenic differentiation. Primary mouse calvarial osteoblasts are also widely used [48], but studies with mouse osteoblasts add some risk of missing human cell-specific signaling.

A second challenge for studying direct effects of osteoblasts on multiple myeloma is that, as with myeloma-derived MSCs [49], myeloma-derived osteoblasts differ substantially from their healthy-donor counterparts [50]. Specifically, their proliferation and osteogenic potential are significantly inhibited and their expression of the CCL3 receptor (CCR1) is significantly increased, which is one pathway contributing to their decreased osteogenic capacity [50]. By studying interactions between normal osteoblasts and myeloma cells, we may not observe the changes that occur in patients, which are between myeloma cells and myeloma-associated osteoblasts. Future studies may more accurately understand the relationships between these cells types if myeloma-patient-derived osteoblasts are utilized.

A third challenge to studying the direct effects of osteoblasts on myeloma cell growth relates to the *in vitro* conditions in which co-cultures are maintained. Most *in vitro* cultures are performed in two-dimensions on flat tissue-culture plates, but models to better mimic the physiologically relevant three-dimensional (3D) nature of the bone microenvironment are now become more established [24,51,52]. Tissue-engineered 3D bone built on silk scaffolds allows for highly reproducible, cost-effective replicates of cultures of osteoblasts and myeloma cells and can be used to model the process of differentiation of MSCs into a mineralized, porous artificial bone environment. These cultures are now being adapted to model the interactions between myeloma cells and any other cells, in the microenvironment, to better elucidate MM-bone stromal cell relationships (unpublished data).

Indirect effects of osteoblasts on myeloma cells through interaction with other cells

Osteoclasts

The most well-documented osteoblast relationship in the bone is the forward-feedback mechanism with osteoclasts known as remodeling. Increased osteoblastic activity leads to increased osteoclastic activity, which can then trigger recruitment of more osteoblasts and vice versa. This cycle is essential for maintaining bone mass and strength [53]. The pathophysiology of myeloma-induced bone disease progressing through the "vicious cycle" occurs when myeloma cells hijack the normal bone remodeling process and skew the balance towards increased osteolytic processes. This state of inhibited osteoblastic activity and increased osteoclastic activity, stimulated through molecules such as RANKL from myeloma cells and osteoblasts [54], leads to osteolytic lesions, weakened bone, pathological fracture, and a release of bone-embedded growth factors that further promote tumor cell growth [2,4,55].

Osteoclasts not only degrade bone matrix to release tumorigenic factors, but also directly promote the survival and proliferation of myeloma cells [40]. Hence, it is possible that use of bone anabolic treatments to increase osteoblastic activity would have a counter effect of also stimulating osteoclastic activity, thereby mitigating the tumor-suppressing effect of newly formed osteoblasts. Similarly, decreasing osteoclastic activities through agents such as bisphosphonates may have the opposite effect, due to the subsequent suppression of osteoblast function *in vivo*, hence diminishing any potential osteoblastic anti-multiple myeloma action. Therefore, understanding the regulation of the timing, location, and responses of osteoclasts to osteoblasts, and the reverse, is crucial for optimizing bone anabolic treatment regimens.

Adipocytes

There is growing interest in understanding interactions between bone and fat cells in normal physiology and disease, and the dynamic relationships between osteoblasts and marrow adipocytes are likely to affect multiple myeloma within the microenvironment in numerous ways. Previously, it was thought that obesity was associated with stronger bones, but more evidence has surfaced that obesity and osteoporosis share common genetic and environmental factors and that excessive fat and obesity may not protect against osteoporosis but could, in fact, accelerate it [56]. The interaction between adipocytes and osteoblasts has traditionally been considered as mutually exclusive such that the transcription factors that induce osteoblastogenesis inhibit adipogenesis and vice versa [56]. Interestingly, there is a significant degree of lineage plasticity between adipocytes and osteoblasts, which share a common progenitor, that further complicates dissecting the relationship between these two cell types in healthy and cancer-containing bone marrow [57,58]. Recent evidence suggests, however, that bone marrow adipocytes may derive from a progenitor cell distinct from the progenitor for osteoblasts, chondrocytes, and other bone marrow stromal cells [59,60].

There are also intriguing data that suggest adipocytes may regulate the pathogenesis and progression of multiple myeloma. A high body mass index (BMI) correlates with increased risk for multiple myeloma [61,62], possibly through increased conversion of androgens to estrogens that in turn stimulate estrogen receptor positive multiple myeloma cells [63–65]. High BMI may also lead to increased multiple myeloma development through increases in inflammatory mediators or CCL2- and COX-2-driven pathways that stimulate tumor growth in the bone marrow [66], but more mechanistic studies are needed to understand these signals. *In vitro* experiments have demonstrated a role for adipocytes in increasing the proliferation of multiple myeloma cells, but whether this is mediated by leptin or other adipokines has not been resolved [67]. Increased bone marrow adiposity in high BMI patients may also support multiple myeloma progression through the disruption of normal hematopoiesis and immune function [68]. In contrast, other reports have shown no difference, or even better overall survival or progression-free survival with certain treatments (e.g., melphalan and total body irradiation) in obese and extremely obese patients compared with normal and overweight patients [69]. Based on preliminary data, it appears that increasing osteoblastic differentiation and activity could decrease myeloma activity in part by decreasing the recruitment of adipocytes within the bone marrow niche, but this remains an open area of research.

Hematopoietic niche and immune cell interaction with myeloma cells

Because the osteoblastic niche is also a site for hematopoietic stem cell (HSC) and immune cell homing and homeostasis [70], osteoblasts may inhibit multiple myeloma growth partially by supporting anti-myeloma immune cell homing to the bone marrow. However, specific types of osteoblasts may play different immune supportive roles, as it appears that only a subtype of osteoblasts, those termed spindle-shaped N-cadherin+/CD45- Osteoblasts (SNOs), located next to the endosteal surface of bone, function to retain the so called Long-Term (LT)-HSCs in a quiescent status [12]. The relationship between osteoblasts and immune cells is complex. For example, although sclerostin null mice have high bone mineral density, they have increased B-cell apoptosis due to decreased osteoblast-derived CXCL12, resulting from increased Wnt signaling [71]. Moreover, although the immune system in general suppresses multiple myeloma [72], not all immune cells

mediate this role. Regulatory T-cells and immunosuppressive myeloid-derived suppressor cells [73] are now being identified as important new targets that inhibit the immune response in multiple myeloma [74]. Interestingly, cellular immunity was found to be decreased in myeloma patients, including decreased ratio of $CD4^+/CD8^+$, DC1/DC2, and Th1/Th2 cells, as well as an increased ratio of regulatory T cells, and some of these metrics of immune function ($CD4^+/CD8^+$ ratio and $CD4^+CD25^+/CD3^+$T ratio) were significantly positively correlated with the quantity of osteoblasts [75]. Hence, the potential effects of osteoblast loss on multiple myeloma via inhibition of the immune system require further investigation.

Other cells in the osteoprogenitor lineage

MSCs, the osteoblast progenitors, and myeloma progression. Bone marrow-derived MSCs are osteoprogenitor cells capable of differentiating into osteoblasts, adipocytes, and chondrocytes, among other cells, and much research has demonstrated their support of multiple myeloma adhesion, growth, and drug-resistance *in vitro* [24–26,76]. The expression of signaling cytokines, extracellular matrix factors, and adhesion molecules is the basis for their important role in myelomagenesis, bone marrow homing, and proliferation [24,77–80]. MSCs from myeloma patients are abnormal in terms of osteogenic differentiation, proliferation, gene expression, and other functions [24,49,81]. By inhibiting osteogenic differentiation of MSCs, multiple myeloma cells may be cleverly retaining a population of cells known to support their survival while inhibiting the maturation of osteoblasts, which generate bone matrix and have suppressive effects on myeloma cells.

In vitro, both osteoblasts and osteocytes can support MSC osteogenesis, in part due to soluble osteogenic cytokines [82]. However, osteoblasts seem to support an initial proliferation of MSCs and a delayed differentiation, while osteocytes promote an initial osteogenic differentiation [82]. However, contrasting the HSC niche roles of osteoblasts, it is less well understood how osteoblasts affect MSC homing, quiescence, and differentiation *in vivo* in healthy bone marrow, and even less so in myeloma-infiltrated bone marrow.

One study found that human placenta-derived adherent cells (PDACs), a type of MSC, inhibit H929 myeloma cell growth in a subcutaneous tumor model (tumor cells grown subcutaneously, later injected with PDACs). However, when the tumor was grown instead in a rabbit bone that was implanted subcutaneously into a SCID mouse (the SCID-rab model), the injected PDACs inhibited growth of H929 myeloma cells [82]. This may indicate that MSCs in multiple myeloma are dependent on the presence of the bone microenvironment to show anti-myeloma effects. Hence, osteoblasts may be essential regulators of the osteoprogenitor phenotype, and they may support a more anti-myeloma phenotype in MSCs.

Osteocytes, the osteoblast descendant, and myeloma progression. Upon becoming encased in osteon, osteoblasts become osteocytes and play a key regulatory role in bone homeostasis, osteoclast activity, and osteoblast regulation. Osteocytes are the mechanosensing cells that reside in lacuna and connect with each other through dendritic processes extending through lacunar-canalicular networks. They have been considered switchboard operators, as they direct a number of different signals that control cells behavior. For example, they extend processes into the vasculature within the bone, and out into the osteoblast-lined surfaces of the marrow and periosteum. With age, these lacunar-canalicular networks become compromised with large sections of bone lacking live osteocytes, suggesting one mechanism whereby diseases that have increasing incidence with age, such as myeloma, may have enhanced growth potential and progression. In several cancers, it has been shown that osteocytes affect tumor evolution through a number of local signaling and endocrine mechanisms [12,83,84].

The relationship between osteoblasts and osteocytes is complicated by the addition of myeloma cells. Since osteoblasts give rise to osteocytes as they become encased in bone matrix, myeloma inhibition of osteoblasts and osteoblastic activity may be a major cause of the decreased osteocytes observed in clinical samples [85]. However, since osteocytes are one of the major producers of sclerostin, a Wnt antagonist, a decrease in osteocytes for any reason typically decreases sclerostin levels, which then stimulates osteoblastic activity to produce a stable bone equilibrium. Unfortunately, the net balance in multiple myeloma patients is osteolysis and loss of osteoblasts/osteocytes; the attempt by the bone to normalize itself is futile and eventually toppled by the burden of osteoclastic activity. For more on the roles of osteocytes in multiple myeloma, refer to the elegant review by Roodman et al. [86].

Canopy-lining cells, the osteoblast cousin, and myeloma progression. Although similar in lineage to active, bone-matrix-secreting osteoblasts, canopy lining cells are quiescent, bone marrow protecting cells. These cells isolate areas of turnover to create a tightly connected, single-cell wide physical barrier to seal-off the osteoclast/osteoblast resorption pit from the marrow [87]. The relatively flat, elongated cells immunostain for osteoblast markers osteocalcin, osteonectin, pro-collagen type I (PINP), pro-collagen type III (PIIINP) and NCAM (CD56), demonstrating that the cell originates from the osteoblast lineage [88]. Importantly, they are Ki-67 negative (hence, non-proliferative) and negative for lymphocytic and monocytic markers. How these cells differ, if at all, from the more classically described quiescent bone lining cells remains to be delineated

Canopy lining cells may play an important role in the dysregulation of bone remodeling in general [89] and could be a novel target cell type in multiple myeloma. Osteoblasts seem to require these cells to properly lay down matrix, as multiple myeloma biopsies analyzed for the presence of these canopies over the bone remodeling compartment (BRC) demonstrated frequent disruptions in 66% of the biopsies. Importantly, frequent disruption (holes) in the canopies correlated with extensive resorption without matrix reconstruction, not observed in biopsies with normal, intact canopies over the BRCs [83,88,90]. Only in multiple myeloma bone surfaces with disrupted canopies did the researchers observe an absence of coupling between bone formation and resorption in patient biopsies [90]. It remains to be determined if BRC canopy destruction in multiple myeloma is a cause or result of deficient bone formation. The microanatomical structures may function through multiple unclear mechanisms (e.g., exerting physical constraints for cells or chemoattractants or acting as anchorage points for certain progenitors), but it is evident that their disruption results in direct physical contact between myeloma cells, osteoclasts, and osteoblasts, and coincides with the occurrence of osteolytic lesions.

Systemic effects

Osteoblasts, osteoclasts, and osteocytes contribute not only to local modifications of bone but also to systemic changes in whole body homeostasis through secretion of specific peptides and growth factors. Traditionally, the action of these cells define the bone as an endocrine organ, responding to hormones and soluble signaling molecules such as estrogen via estrogen receptor α (ERα) [91], calcium, PTH, 1, 25-Dihydroxycholecalciferol, and vitamin D, which communicate with other endocrine organs throughout the body, such as the thyroid, parathyroid, pituitary glands, adrenal glands, and pancreas, as reviewed elsewhere [92]. More recently, the secretion of metabolically active peptides such as osteocalcin has been shown to regulate insulin sensitivity and secretion. During states of high bone turnover, the release of matrix and cell-derived undercarboxylated osteocalcin impact adipose tissue sensitivity to insulin which in turn could release adipokines that further modulate myeloma progression [93]. On the other hand, bone metabolism may appear normal, as judged by biochemical measurements such as urinary excretion of calcium, hydroxyproline, and n-telopeptide, but significant bone destruction may be present. Hence, experiments to alter the local bone milieu, to dissect the roles of osteoblasts on

myeloma growth, must be performed in conjunction with monitoring other systemic changes resulting from alterations of osteoblasts. Measuring bioactive factors that are liberated during bone destruction may help quantify bone turnover, but cannot be used as a definite readout, due to a variety of confounding systemic effects resulting from osteoblast stimulation or inhibition [6].

In vivo models of osteoanabolism as a therapeutic approach to multiple myeloma

Osteoanabolic treatment is defined as any treatment that stimulates osteoblastic activity and bone formation. As a therapy for multiple myeloma, this strategy has yielded conflicting conclusions, with *in vivo* efficacy depending on the model system and treatments used. Some mouse models, such as the patient xenograft SCID-hu model, demonstrated that osteoanabolic treatments hold promise for inhibiting multiple myeloma, although results were highly variable and patient-specific [40]. There is mounting evidence that the anti-myeloma proteasome inhibitors carfilzomib [94] and bortezomib [95] have bone anabolic effects on bone and induce osteogenic differentiation of MSCs, which may contribute to their anti-myeloma effects [96], but concrete evidence remains elusive to demonstrate that these agents can produce anti-myeloma effects via changes in the bone microenvironment. The use of anti-resorptive agents, such as bisphosphonates, cathepsin K inhibitors, or RANKL inhibitors [54], in combination with osteoanabolic agents, may maximize the use of the bone microenvironment to inhibit myeloma. Collectively, these results highlight the need for better *in vivo* models and deeper understanding of exactly how we predict osteoanabolic treatments may function to inhibit multiple myeloma.

Anti-sclerostin and anti-DKK1 antibodies also have osteoanabolic effects in preclinical models and in clinical trials and are currently under investigation for the treatment of osteoporosis and osteolytic disease [97,98]. Anti-sclerostin treatments may prove useful for osteolytic cancers in general, but especially for myeloma, since myeloma cells secrete sclerostin that inhibits osteoblast activity [99]. Anti-DKK1 treatments may also be viable mechanisms for inhibiting myeloma bone disease, as DKK1, a canonical Wnt pathway inhibitor, is overexpressed in myeloma cells and patient serum [100], and DKK1 levels correlate with the extent of lytic bone disease [101]. Anti-DKK1 antibody therapy has also been shown to significantly increase osteoblast bone formation and bone mineral density in both murine and human healthy and multiple myeloma models [97,102,103]. Anti-DKK1 therapy in myeloma also inhibits osteolysis in multiple myeloma SCID-rab models (SCID mouse with rabbit bone subcutaneous implantation) [104]. However, the rates of success at lowering IgG levels or decreasing tumor growth rate, measured by tumor size, were only 36% (4/11) and were patient specific, suggesting that bone anabolic treatments may work only for a subset of myeloma patients [104]. A different myeloma model used to test anti-DKK1 antibodies is the SCID-hu model with fetal bone chips in a SCID mouse, injected with INA-6 myeloma cells. In this model, treatment with the Novartis antibody BHQ880, which neutralizes both human and murine DKK1, promoted osteoblastogenesis and decreased tumor burden, as measured by *in vivo* IL6 levels [102].

Other bone anabolic agents, including dasatinib, a multitargeted tyrosine kinase inhibitor [105], and soluble decoy receptors of activin A, a known osteoclast activating factor [106], also inhibit multiple myeloma, suggesting their clinical utility and supporting the hypothesis that increasing bone volume and osteoblast number is a practical method for inhibiting multiple myeloma [107,108]. Similarly, TGF-β, a potent inhibitor of terminal osteoblast differentiation abundant in the bone matrix, has also been identified as a novel target. Anti-TGF-β therapies are able to restore osteoblast differentiation suppressed in MM conditions *in vitro* and suppress myeloma cell growth within the bone marrow (using the SCID-rab/INA6 myeloma model) while preventing bone destruction in myeloma-bearing animal models [27]. This study demonstrated that osteoblasts, defined as mineralized MC3T3-E1 cells, were able to induce apoptosis and G1 cell cycle arrest in 5TGM1 myeloma cells, although the exact mechanisms by which osteoblasts potentiated these effects were not explored [27].

In vivo studies using daily administered parathyroid hormone (PTH) in SCID-rab and SCID-hu mouse models demonstrated that PTH treatment increased bone mineral density and reduced tumor burden [109]. PTH also increased the number of osteoblasts and other bone formation parameters and pre-treatment with PTH before injecting tumor cells also increased bone mineral density and delayed tumor progression. This research supports the hypothesis that an increase in bone mineral density and osteoblast number may provide a net anti-myeloma effect. Importantly, PTH can clinically lead to increased bone formation and osteoblast activity within the first 6 months of treatment. However, with longer-term PTH administration, osteoblast activation slows, and importantly, bone resorption increases significantly. Theoretically, this could compromise any positive effects of this approach for slowing myeloma progression [110]. Thus, osteoanabolic therapies for bone utilize a range of different approaches and target pathways, nicely summarized in a recent review [111]. However, it still remains controversial whether reported anti-tumor effects of bone-modulating therapies are clinically significant [112]. The current challenge in myeloma therapeutics thus becomes not only to develop biologic agents that have the desired effect of killing cancer cells but also to prevent any rebound or compensation that could make the skeletal changes worse.

Clinical studies have shown that the treatment of multiple myeloma patients with bisphosphonates significantly overall survival and progression-free survival [113,114]. Zoledronic acid and bortezomib both have anti-myeloma effects. Zoledronic acid is thought to directly impact myeloma cells, and bortezomib additionally may induce "pro-bone" mechanisms, including increasing osteogenic differentiation and inhibiting osteoclasts [95,115]. In fact, based on these studies, a phase II clinical trial recently completed in smoldering multiple myeloma patients treated with low dose bortezomib had a primary endpoint, "to evaluate the bone anabolic effect of bortezomib in patients with smoldering myeloma" and a secondary endpoint "to evaluate the effect of bortezomib on the natural history of smoldering myeloma" [116]. Another interesting phase II trial in the recruitment stage aims to test Sotatercept, an activin-A antagonist that interferes with the SMAD pathway. This signaling network, when activated, can lead to increased bone formation and anti-tumor activity in multiple myeloma [117] and bone anabolic improvements in bone mineral density and in bone formation [118]. The effects of Sotatercept on patient-specific outcomes such as skeletal-related events (i.e., fractures, impaired healing, bone pain) as well as delayed-progression, or progression-free or overall survival, remain to be elucidated. Results from these ongoing and future trials may open the door for similar treatments to be tested in patients with early stage or even overt myeloma.

Future directions and conclusions

One of the new directions in osteoblast-myeloma research, and in tumor-host interaction studies in general, is the use of CRISPR-Cas9 knockout technologies [119]. With this technology, researchers have already demonstrated an ability to more specifically target genes such as Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3) in myeloma and hence dissociate the anti-tumor and teratogenic activities of thalidomide-like drugs [120]. The use of CRISPR technologies for modulating host osteoblasts and bone marrow cells would provide abundant information regarding the roles of different genes in bone cells and could suggest novel mechanisms for modulating the bone microenvironment to induce a less hospitable environment for the growth of cancer cells. Also, if investigators can overcome the obstacles and potential off–target effects of microRNA delivery, these may be a potential novel osteoanabolic treatment. "Osteogenic microRNAs" have been identified [121] and are currently under investigation for *in vivo* efficacy. Interestingly, some of these have been identified as differentially expressed in

multiple myeloma versus healthy MSCs and capable of functionally rescuing MSCs for their ability to produce bone matrix [24].

Multiple myeloma is considered by some a prototype for metastatic bone disease although there are clear phenotypic distinctions from other malignancies such as prostate cancer. Nevertheless, studies of osteoblastic function in myeloma could be extrapolated to other conditions that have classically been considered osteolytic, such as metastatic breast cancer [122–125]. This review described the multitude of ways in which osteoblasts may function to support or inhibit myeloma growth, and discussed new potential targets in the relationship between osteoblasts and myeloma cells to treat or prevent multiple myeloma. Osteoblasts act as an important hub of activity, affecting other cells within the bone marrow niche and mediating both direct and indirect effects on myeloma cells (Fig. 1). The future of bone anabolic treatments for anti-myeloma therapy is bright, but to optimize the use and design of such agents, it will be critical to view the osteoblast within a larger context and to visualize its interactions with other cells in the bone microenvironment and roles in whole body homeostasis.

Author contributions

MRR conceived and wrote the manuscript; LL designed and created the figure; LL, CJR, and IMG provided intellectual input and edited the manuscript.

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Conflicts of interest

The authors declare no competing financial interest. Ghobrial: *BMS*: Advisory Board; *Celgene*: Advisory Board, Advisory Board Other; *Millennium*: Advisory Board, Advisory Board Other; *Onyx*: Advisory Board, Advisory Board Other.

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Inside the Institute

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Reardon receives brain tumor research grant p. 2

Hassett discusses benefits of Epic implementation p. 3

Employee raises awareness to honor sister p. 4

Administrative fellows develop leadership skills

Before last summer, Marian "Manny" Hill had never visited the Dana-Farber campus. Now, just a year after college, she's participating in meetings with the Institute's top leaders and learning skills needed to build a health care management career.

What is the secret of her success? Hill is part of what has become one of Dana-Farber's most successful avenues for advancement: the Administrative Fellowship Program (AFP). Established in 2007, the one-year program allows individuals to develop their leadership style and analytical skills while gaining expertise in such areas as Finance, Human Resources, Patient Care Services, Research Administration, and Clinical Operations.

Selected from more than 100 candidates with master's degrees in health care administration, business administration, public health, or related fields, Hill – who has a master's in biomedical engineering from the University of Michigan – started her fellowship on July 1 and is spending her first two months shadowing executive

Fellows, *page 4*

Fellowship gives students inside look at oncology nursing

Kayla Costigan grew up admiring her mother, an oncology nurse for more than 20 years. Lauren Guerra remembers reading to her toddler cousin in the hospital during his cancer treatment.

Rising seniors at Boston College, both Costigan and Guerra have long had a passion for cancer care. Now, thanks to a new program at Dana-Farber, they are getting a glimpse at adult oncology nursing.

Through a fellowship program made possible by

Oncology nursing, *page 4*

Lauren Guerra (left) and Kayla Costigan are participating in an oncology nursing fellowship program established by Frederick C. Flynn Jr. (center) in honor of his late wife.

Michaela Reagan (left) and Irene Ghobrial are studying a delivery system using nanoparticles that target bone cancer cells and release a drug to slow the progress of multiple myeloma.

Nanoparticles deliver drugs to fight bone cancer

Scientists have demonstrated in mice a delivery system using drug-filled microscopic nanoparticles that home in on bone cancer cells and release the drug to slow the progress of multiple myeloma. They report that the treatment enhanced the strength and volume of the bone as well.

Such a system could be used to treat patients with multiple myeloma or other cancers that metastasize to the bone, say researchers from Dana-Farber and Brigham and Women's Hospital (BWH) who collaborated on the project. A report on the study was published June 30 in *Proceedings of the National Academy of Sciences*.

"There are limited treatment options for bone cancers," notes Michaela Reagan, PhD, of Dana-Farber's Center for Hematologic Oncology, co-lead study author. "Our engineered targeted therapies manipulate the tumor cells in the bone and the surrounding microenvironment

Nanoparticles, *page 3*

Fat cell protein signal found to stimulate insulin production

Researchers have shown in diabetic mice that a protein made by fat cells is critical to the normal production of insulin, the hormone that enables the body to maintain a healthy level of blood sugar.

Dana-Farber's Bruce Spiegelman, PhD, senior author of the report in *Cell*, says the newly discovered role of the protein, adipsin, could have implications for treatment of type 2 diabetes, a growing epidemic affecting an estimated 382 million people globally.

In an experiment with obese, diabetic mice that lacked adipsin, restoring the protein to the animals improved the health of beta cells in the pancreas. Beta cells secrete insulin, which helps muscle cells use sugar (glucose) for fuel. The beta cells malfunction in severe diabetes.

The scientists also discovered that adipsin is deficient in human patients with severe type 2 diabetes.

"This suggests a new approach to treating type 2 diabetes in patients whose pancreatic beta cells work poorly, leaving them dependent on injected insulin," says Spiegelman, of the Department of Cancer Biology.

"If humans respond similarly to the mice in this study," he continues, "correcting their deficiency of adipsin would improve beta cell function and perhaps maintain enough natural insulin production to avoid or delay the need for insulin injections."

Per-Olof Berggren, PhD, of Karolinska Institutet in Stockholm, Sweden, and a co-investigator of the study, adds that adipsin "might be the long-sought molecule linking fat tissue metabolism to pancreatic beta cell function." James Lo, MD, PhD, a cardiologist in the Spiegelman lab, is the report's

Fat cell protein, *page 3*

News of Note


David Reardon

Novel immunotherapies to be tested in brain tumor patients

With the support of a three-year grant from the Ben and Catherine Ivy Foundation, Dana-Farber researchers are preparing a multi-pronged attack on glioblastoma brain tumors with a combination of immunotherapy methods.

A small pilot clinical trial of a cutting-edge vaccine could begin enrolling glioblastoma patients later this year, according to **David Reardon, MD**, principal investigator on the $1.5 million grant from the foundation, which funds research to improve the survival of patients with these aggressive brain tumors. Reardon is the clinical director of Dana-Farber's Center for Neuro-Oncology.

The vaccine, called NeoVax, was developed by a research team at Dana-Farber and the Broad Institute of MIT and Harvard led by Cathy Wu, MD. NeoVax is tailored to each patient's tumor cells that carry on their surface highly specific identifying molecules created by mutations that are unique to each patient's glioblastoma. The vaccine treatment is designed to "teach" the patient's immune system to identify and react against those cancer cells. NeoVax is currently being tested at Dana-Farber in patients with advanced melanoma.

In a second project, carried out in collaboration with David Mooney, PhD, and other colleagues at the Wyss Institute, researchers will test an implantable vaccine delivery device that "reprograms" the immune system to generate an immune attack on glioblastoma cells.

The third part of the research focuses on how glioblastomas, like many other tumors, shield themselves from the body's immune defenses, which otherwise would attack the cancer. Drugs based on discoveries by Gordon Freeman, PhD, of Dana-Farber, have showed great promise in some forms of cancer by blocking the "immune checkpoints" that shield the tumors from attack. With the Ivy grant support, scientists will continue studies in mice in which these drugs achieved long-lasting elimination of glioblastomas.

The ultimate goal is to combine these three approaches into a unified treatment approach that can generate long-term anti-tumor activity and potentially eradicate glioblastomas, says Reardon. RS


Andrew Wagner

Wagner receives grant for liposarcoma research

The Liddy Shriver Sarcoma Initiative has selected **Andrew Wagner, MD, PhD**, for an international team grant aimed at improving the understanding and treatment of liposarcoma, a rare form of sarcoma. The two-year, $250,000 International Collaborative Grant is funded in partnership with The Wendy Walk.

The Liddy Shriver Sarcoma Initiative supports research on the estimated 50 different types of sarcomas. It is named in honor of Liddy Shriver, daughter of Bruce and Bev Shriver, who passed away at age 37 after battling Ewing sarcoma. The Wendy Walk was formed by the children of sarcoma patient Wendy Landes, who fought liposarcoma and passed away in March 2013.

Wagner will work with three other scientists – two from Norway and one from Australia – to identify genetic factors that distinguish two forms of liposarcoma. One form of the disease grows gradually and is not a danger to spread, while the other form is more aggressive and can quickly become fatal.

"I'm thrilled to be joining this group of outstanding researchers and collaborators in our approaches to improving our understanding and treatment of liposarcoma," says Wagner. If the research pinpoints genes that trigger the change to the more aggressive form, "we will potentially be able to test drugs that specifically target these alterations," Wagner says. MG

Dana-Farber researchers awarded Melanoma Research Alliance grants

Seven Dana-Farber doctors were awarded research grants by the Melanoma Research Alliance (MRA). MRA is the largest private funder of melanoma research and this year awarded a record-breaking $8 million in new research grants.

MRA awards research grants to "both individual investigator and collaborative team projects focused on translational, innovative research that will impact the prevention, diagnosis, staging, and treatment of melanoma in the near and immediate future," the award statement reads.

Dana-Farber doctors were awarded three Team Science Awards and one Academic Industry Award. **F. Stephen Hodi, MD**, received the Academic Industry Award, one of three awards offered annually. This award is unique because it "represents a novel mechanism for collaboration among academic researchers, industry, and MRA through a corporate match of MRA's funding," according to MRA.

Team Science Award recipients were chosen based upon the goal of the award, "to foster a collaborative research process that promotes transformational melanoma research advances with the potential for rapid clinical translation." They include **Loren Walensky, MD, PhD; Levi Garraway, MD, PhD; James Bradner, MD; Jason Luke, MD; Kai Wucherpfennig, PhD;** and **Michael Goldberg, PhD**.

"These grants are infused with MRA's spirit of collaboration, reflecting geographic diversity and drawing from many scientific disciplines," says MRA co-founder and chair Debra Black. "Together, we are making huge strides against this disease." WE

Jon Lester jersey giveaway

For more than 60 years, the Red Sox have had a special bond with the Jimmy Fund and Dana-Farber Cancer Institute – the longest and most successful partnership between a professional sports team and charity in North America. The Red Sox have helped the Jimmy Fund raise millions of dollars for cancer care and research at Dana-Farber through appearances and appeals, while befriending patients of all ages.

In honor of this partnership, we're giving away a signed Jon Lester jersey to one lucky fan. Encourage your patients and friends to vote for their favorite Red Sox star on the Dana-Farber Facebook page to be entered to win.

Contest ends at 11:59 p.m. on Tuesday, August 12. Dana-Farber employees and their immediate family members are not eligible to win.

New MASCO shuttle buses on the road

If you're a frequent rider of the MASCO shuttles, you may have noticed a recent upgrade to the bus fleet.

On June 30, MASCO rolled out 30 new large shuttle buses. The clean diesel buses run on all MASCO shuttle routes and feature quieter engines, controlled interior temperature, and comfier seats.

In addition to the 30 buses, MASCO has also purchased seven smaller vans for its shuttle routes. The vans will hit the road in fall 2014.

"MASCO is committed to providing safe, comfortable, and timely transportation to our riders," says Marilyn Swartz-Lloyd, president and CEO of MASCO. "They are hard-working, mission-driven employees, so it is extremely important to us that we provide our riders the very best in comfortable, efficient, and safe transportation," she said.

MASCO purchased the new fleet using a $13.4 million tax-exempt lease through JP Morgan. The lease was made possible through a partnership with MassDevelopment, the state's finance and development agency. MG

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Story ideas are welcome and can be sent to Naomi Funkhouser at 450 Brookline Ave., OS301, Boston, MA 02215. You can also call 617-632-5560, fax to 617-632-5520, or email *Naomi_Funkhouser@dfci.harvard.edu*. Visit the Dana-Farber website at ***www.dana-farber.org*** or the intranet at ***www.dfcionline.org.***

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Research takes aim at cancer's genetic 'addictions'

Breaking an addiction to illegal drugs can bring a chance at a new life. Breaking a cancer cell's addiction to certain growth-spurring genes can spell the cell's death.

In some cases, that's a relatively straightforward task: design a drug that can block the abnormal gene from continually issuing growth commands. But what if that gene isn't abnormal – harbors no mutations – but has been whipped into hyperactivity by some other actor within the cell?

If so, proteins called transcription factors may be the culprits. They control how genetic information is converted to a ready-to-use form, and thus serve as on-off switches for genes. Unfortunately, transcription factors are notoriously difficult to target with drug compounds.

It's now known that transcription factors don't work solo; they often rely on "co-factor" enzymes to perform their function. While these co-factors bring an additional level of complexity to the transcription process, they also offer an enticing array of targets for new therapies. Blocking a co-factor with a drug could, in theory, disable a key transcription factor, thereby shutting down a gene relentlessly sending growth signals to the cell.

In new research published in the journal *Nature*, scientists at Dana-Farber and other institutions succeeded in killing laboratory-grown leukemia cells with this type of molecular sneak attack. They used an agent that, instead of directly targeting a co-factor, targets an exquisitely sensitive part of the genome that has an outsize influence on the co-factor's ability to function. It represents one of the first times an enzymatic co-factor has been stymied with this technique.

"We've known for many years that some cancer cells are dependent on the continual transcription of certain genes. Without the constant growth signals sent by these genes, such cells won't survive," says Dana-Farber's Nathanael Gray, PhD, the study's senior author. "In this study, we've used a novel technique to disrupt such transcription and bring about cancer cell death."

Previous research had shown that T-cell acute lymphocytic leukemia (T-ALL) cells are highly susceptible to a drug compound called THZ1. In the current study, Gray and his colleagues set out to discover why this is so.

They found that THZ1 latches onto a long DNA segment known as a "super-enhancer" because of its exceptional power over gene activity. About 10 times the size of standard enhancers, and with a jumble of proteins attached, super-enhancers are the giants of the cell's gene-controlling machinery. But like the biblical giant Goliath, super-enhancers are not only massive, they're also highly vulnerable, easily disrupted with a pinpoint strike.

That's what THZ1 accomplishes, Gray and his colleagues discovered: it disables a super-enhancer by blocking an enzymatic co-factor called CDK7. That, in turn, halts transcription of the genes *MYC* and *RUNX1*, which are overactive in the T-ALL cells. The result is death of the leukemia cells.

Gray notes that while the technique has worked admirably in laboratory cell cultures, more work is needed to determine if it can succeed in patients without producing severe side effects. RL

Fat cell protein, *continued from page 1*

first author.

Checking for adipsin levels in diabetic patients might help doctors predict which individuals are at highest risk of impending beta cell failure so they can begin treatment earlier, the authors note.

Adipsin was the first of a class of proteins called adipokines to be discovered. These proteins, which are secreted into the bloodstream by adipocytes, or fat cells, continuously circulate to influence a variety of metabolic and immune functions. Spiegelman's research group discovered the protein and its connection to the immune system in 1987, but its newly identified role in controlling insulin production by the pancreas was entirely unsuspected.

The Spiegelman lab and other researchers have recently found some immune system components are present in fat cells, where they help maintain the body's energy balance. This growing area of investigation prompted Lo and Spiegelman to revisit the function of adipsin. Lower levels of adipsin had been reported in obese and diabetic animals, but overweight and diabetic humans experienced unchanged or elevated levels, leaving it unclear how the protein functions in those conditions.

In this study, the Dana-Farber investigators used "knockout" mice lacking the adipsin gene and "wild type" mice with normal adipsin levels. Both sets of animals became obese on a high-fat diet and developed excess blood sugar – a pre-diabetic state. The symptoms were worse in the adipsin knockout mice.

In the report, the scientists say the difference in symptoms is explained by "an unexpected and striking requirement of adipsin for proper insulin secretion by the pancreatic beta cells." RS

Nanoparticles, *continued from page 1*

to effectively prevent cancer from spreading in bone with minimal unwanted effects."

Reagan is a postdoctoral fellow in the laboratory of Irene Ghobrial, MD, a medical oncologist in the Jerome Lipper Multiple Myeloma Center at Dana-Farber/Brigham and Women's Cancer Center and co-senior author of the study with Omid Farokhzad, MD, director of the BWH Laboratory of Nanomedicine and Biomaterials.

"Bone is a favorable microenvironment for the growth of cancer cells that migrate from tumors in distant organs of the body, such as breast, prostate, and blood, during disease progression," explains Archana Swami, PhD, of the Laboratory of Nanomedicine and Biomaterials. She is a co-lead author of the study.

"We engineered and tested a nanoparticle system to selectively target the bone microenvironment and release a therapeutic drug in a controlled manner, leading to prevention of disease progression," says Swami.

The scientists developed nanoparticles made up of biodegradable materials, coated with alendronate, a type of drug that binds to calcium. The mice were pre-treated with nanoparticles loaded with the anti-cancer drug bortezomib before being injected with myeloma cells. The treatment resulted in slower myeloma growth and prolonged survival of the mice. Moreover, the researchers also observed that bortezomib, as a pre-treatment agent, changed the makeup of bone, enhancing its strength and volume.

"This study provides the proof-of-concept that targeting the bone marrow niche can prevent or delay bone metastasis," says Ghobrial. "This work will pave the way for the development of innovative clinical trials in patients with myeloma to prevent progression from early precursor stages, or in patients with breast, prostate, or lung cancer who are at high risk of developing bone metastasis." RS

An **Epic** Journey

A conversation with Michael Hassett

Michael Hassett, MD, MPH, assistant professor of medicine and medical oncologist in the Breast Oncology Program at the Susan F. Smith Center for Women's Cancers at Dana-Farber, sat down with the Epic group to discuss the Epic implementation and how it will positively affect Dana-Farber and the entire Partners community.

How are you involved with the Epic project?

I am assisting with the DFCI Epic implementation and serve as the clinical lead for oncology for the Partners Clinical Content Team. I work with the Epic Systems Corporation and the Partners organization to collaborate on new developments for the Epic system. I also oversee Epic's oncology module, Beacon [used for cancer staging and chemotherapy treatment plans].

How do you envision the Epic implementation impacting the fight against cancer?

Although DFCI operates on a variety of IT systems that do their job very well, the specialization of each has led to an overall weaker system. With the Epic implementation, we are transitioning to a strong, system-wide infrastructure that will help with care coordination across providers and sites. For instance, providers will be able to see test results and notes from all clinicians regardless of the setting's inpatient or outpatient status.

A core value of DFCI is "Discovery." How will this project help further research efforts?

Because Epic will provide more coordination of care, there is hope that we will be able to better identify patients eligible for clinical trials. Epic also contains a large number of structured data fields, thereby giving DFCI the ability to further discovery by using patient data for approved clinical research.

Do you have any advice for your colleagues for how to thrive in this time of change?

This is going to be a challenging transition, and we all must be prepared, open, and patient. Preparation depends on completion of all necessary training and pre-conversion prep work. We should be open to doing things differently, as our current applications will differ from those we are adopting. Lastly, patience will be key during implementation; this will be a change for the better. MB

To read a transcript of the full interview, visit **http://dfcionline.org/partnersecare** and click on "Resources."

DANA-FARBER CANCER INSTITUTE | Partners *e*Care

DFCI nurse supports research, raises awareness in memory of her sister

By 2003, Eileen Lind, RN, MSN, CPNP, had several years of experience handling treatment protocols and diagnoses, and delivering difficult information to patients and families as a pediatric nurse practitioner at Dana-Farber/Boston Children's Cancer and Blood Disorders Center.

But those experiences would hardly prepare her for the news that her older sister, Maureen Russo, had cervical cancer.

"I thought because I deal with cancer on a regular basis that I could handle her diagnosis, but the dual role of caretaker and oncology nurse was one of the most difficult challenges I've ever faced," Lind says.

Her sister, with her infectious laugh and warm personality, was just 34 years old when she was diagnosed. She sought treatment with Ursula Matulonis, MD, medical director of the Gynecologic Oncology Program at the Susan F. Smith Center for Women's Cancers at Dana-Farber, who worked with Russo through every step of treatment.

"Dr. Matulonis made Maureen feel like she was her only patient; she never made her feel rushed and she was always available to listen to her concerns," Lind says.

After chemotherapy and radiation, Russo eventually

Eileen Lind holds a photograph of her sister, Maureen Russo, who died in 2006 after a three-year battle with cervical cancer.

went into remission, but three years later the disease came back. In December 2006, just six months after her cancer returned, Russo passed away.

In the years since her death, Lind has maintained her relationship with Matulonis, teaming up to raise awareness for cervical cancer prevention and research. Last fall, Lind and Matulonis organized the first annual HPV/Cervical Cancer Summit, held in November 2013. Hosted by the Susan F. Smith Center in collaboration with the American Cancer Society, the event brought together more than 100 public health officials to discuss the expansion of community cervical cancer prevention programs. This year's summit will be held on November 7.

Lind and her family have also worked to keep Russo's spirit alive through Team Maureen, a nonprofit organization that began as a Pan-Mass Challenge (PMC) team. The organization aims to improve the lives of women affected by gynecological cancers by raising money for cancer research and community outreach.

This year, 13 cyclists will join Team Maureen for the PMC, including Russo's husband, Mike.

"It's exciting to see the money we raise support Dana-Farber researchers," Lind says. "The research makes a real difference and helps develop treatments that give patients more time than my sister had."

Lind and Team Maureen also work with Dana-Farber's Community Benefits Program and the Department of Public Health to spread awareness about cancer prevention. Joined by Russo's daughter, Gabrielle, Lind and other health professionals travel throughout the state to educate people, particularly teens, about HPV vaccines, early detection, and screening opportunities.

The goal of these programs, Lind says, is to keep her sister's spirit alive, while reducing the stigma around cervical cancer and educating as many people as possible about how to prevent the disease.

"If we can get away from the stigma and focus on prevention, we can really make a difference," Lind says. MG

Oncology nursing, *continued from page 1*

the Susan D. Flynn Oncology Nurse Training and Development Fund, Costigan and Guerra spent June and July training alongside veteran nurse preceptors Maura Dacey, RN; Erin Drury, RN; Suzanne Oliver, RN; and Michaelle Renard, MS, RN, at Dana-Farber/Brigham and Women's Cancer Center (DF/BWCC). Frederick C. Flynn Jr., a retired business executive, established the fund in honor of the nursing care his late wife, Susan Flynn, experienced before dying of ovarian cancer in May 2013.

Although both students have completed clinical rotations with other students and one nursing faculty member, Costigan and Guerra say the opportunity to work one-on-one with oncology mentors and develop relationships with patients has confirmed their career aspirations.

"We're not exposed to oncology much in our school training," says Costigan. "Being able to watch what veteran nurses do, you learn so much about connecting with families. My mom is quizzing me to see what I've learned."

Like Costigan, Guerra has learned about many specialized areas of oncology nursing, including social work, spiritual care, radiation oncology, integrative therapies, and palliative care, and even observed an operation. With input from their preceptors, they also created presentations – Costigan on the prevention and treatment of chemotherapy-induced nausea, and Guerra on the role of palliative care in pain management and quality of life.

"Everybody on the staff has been willing to teach us, and the patients have been wonderful," says Guerra. "What surprised me the most was how the positives about the cancer care experience far outweigh the negatives. The patients feel it's their job to get better, and as a nurse you focus on helping them get there."

This is the message Clinical Nurse Specialists Anne Elperin, MSN, ANP-BC, AOCNP, and Mary Lou Siefert, DNSc, AOCN, envisioned when coordinating the program. "We're socializing them to the role of an oncology nurse at a comprehensive cancer center, and preparing them for the situations they will encounter in that role," says Elperin. Siefert, noting DF/BWCC has never offered such a program before, adds, "What makes it such a rich experience is that they are able to observe patients along the entire continuum of care."

For Flynn, it's also a lasting legacy to his wife: "I hope the fellowship can better equip and inspire participants to pursue a career in this critical field and give DF/BWCC's next generation of oncology nurses valuable exposure to the mission." SW

Fellows, *continued from page 1*

leadership across the Institute. This inside access to decision-making at a major cancer center is invigorating for Hill, who still remembers her mother's battle with breast cancer during Hill's high school and undergraduate years.

"That experience drew me to the field of biomedical engineering, so I could improve the lives of those with cancer," explains Hill. "When I heard about the mission and culture of Dana-Farber, and then the AFP, it really resonated. I felt driven to apply."

Pictured left to right are Julie Bryar Porter and Mary Tyson, both former administrative fellows, with current fellow Marian Hill.

In doing so, Hill joins a list of past fellows that includes many who have gone on to full-time positions at the Institute. Julie Bryar Porter spent her AFP year of 2010-2011 helping transition patient clinics from the Dana building to the Yawkey Center, overseeing documentation for a Department of Public Health survey, and organizing daily huddles for leadership after the move. Now she is manager of Quality and Patient Safety.

"The AFP confirmed to me that health care management is almost always about the people, especially making sure you have the right staff involved in a decision or a job," says Porter.

Remembering how generous senior leaders were to her as a fellow, Porter enjoys helping others in the role. This feeling of "paying it forward" is shared by Hill's immediate AFP predecessor, Mary Tyson, who has transitioned into a full-time role as manager of clinical strategy in Clinical Planning and Network Operations.

"As a fellow, I had exposure to the highest level of executive decision-making, and was also given the flexibility to gain hands-on analytic and project management experience as a contributing member of project teams," says Tyson. "This balance of observation and active contribution was an incredible learning opportunity."

And a great indicator of future success.

"From the Institute's perspective, we get to see young talent in action," says David Read, vice president of Ambulatory Practice Management and chief administrator for Medical Oncology, who supervises the AFP recipients. "Our administrative fellows are then ready for more senior level leadership positions sooner. Hopefully, if we can convince them to stay at Dana-Farber, their ramp-up time is much shorter." SW

MAGNET RECOGNIZED
AMERICAN NURSES CREDENTIALING CENTER

Dana-Farber designated as Magnet® organization for third time

Dana-Farber was recently reaccredited with Magnet® status from the American Nurses Credentialing Center (ANCC). This prestigious designation recognizes excellence in nursing and health care delivery and has been given to only 7 percent of hospitals nationwide. In 2005, Dana-Farber became the first cancer center in New England to receive Magnet status. We were redesignated in 2009.

"The ANCC Magnet credential recognizes excellence in nursing care delivery, innovation, and evidenced-based practice," says Patricia Reid Ponte, RN, DNSc, FAAN, NEA-BC, Dana-Farber's senior vice president for patient care services and chief nursing officer. "It highlights the exquisite nature of our interdisciplinary practice model and demonstrates, through quality, workforce, and organizational outcomes, that our practice is outstanding, while constantly striving for improvement. It is an honor for me to be working alongside such extraordinary nurses."